Universidade do Minho
Escola de Ciências da Saúde

Maria Inês da Cunha Doutel de Almeida

microRNAs in Cancer: Biological Effects of microRNAs in Colorectal Cancer

microRNAs em Cancro: Efeitos Biológicos de microRNAs no Cancro Colorectal

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Tese de Doutoramento em Ciências da Saúde

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(Maria Inês da Cunha Doutel de Almeida)

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Summary / Resumo

Summary

Non-coding RNAs are a class of RNA transcripts that do not codify for protein. Included in this class are the microRNAs, small RNAs with approximately 20 nucleotides in length. microRNAs act post-transcriptionally by binding to 5´-ultraconserved regions, coding regions or 3´-ultraconserved regions of messenger RNAs and inhibit its translation or cause messenger RNA cleavage. Additionally, microRNAs can also bind to pseudogenes and are involved in RNA decoy functions. A single microRNA can bind to several RNAs and a single RNA can be targeted by different microRNAs. Therefore, microRNAs constitute master regulators of gene expression and major key players in the control of cellular functions.

microRNAs are deregulated in all tumor types and can act as tumor suppressors or as oncogenes in a tissue- and cell-specific manner. microRNAs control biological mechanisms essential for cancer cells, such as proliferation, cell death, cell cycle, metabolism, hypoxia, angiogenesis, inflammation, migration, invasion, metastasis and cancer stem cells self-renewal. Besides microRNAs´ importance as diagnostic and prognostic tools in cancer, and due to their involvement in cancer biology, microRNAs have been regarded as new therapeutic targets. This thesis focuses on the role of microRNAs on colorectal cancer. Despite the great improvement in therapeutics, colorectal cancer is still one of the most commonly diagnosed tumor types and a frequent cause of cancer-related death. We intend to understand the biological effects of miR-28 in this tumor type. To achieve this goal both *in vitro* and *in vivo* approaches were used.

In the microRNA biogenesis pathway, usually a primary-microRNA is processed into a precursor-microRNA, which originates a single mature microRNA. However, in some cases, two mature miRNAs are processed. This occurs with the precursor-miR-28 that is processed into two mature microRNAs that were named miR-28-5p and miR-28-3p. We identified for the first time that both mature forms of miR-28 were deregulated in colorectal cancer. Contrary to some tumor and cell types in which miR-28 was found

to be upregulated, in colorectal cancer miR-28 is downregulated in tumor compared with normal colorectal tissue. However, the two mature forms have distinct functions. Our *in vitro* results revealed that miR-28-5p overexpression decreased proliferation, increased apoptosis, caused a G1-arrest in the cell cycle, and decreased migration and invasion, while miR-28-3p overexpression had no effect on proliferation but increased migration and invasion. This was the first description of miR-28 dual role in colorectal cancer cells. As miR-28 mature forms are transcribed together, we analyzed the overall result *in vivo*. Xenografts of colorectal cancer cells overexpressing miR-28 caused a slower tumor growth in mice, while increased metastasis, when compared with the control group. In this thesis we also discuss the strategies to inhibit or restore microRNAs function. The dual role of miR-28 has great impact on the design of therapies using microRNAs, in particular for upregulation of microRNA levels using expression vectors.

Variation in expression levels of microRNAs has a tremendous impact on the intracellular signaling networks. We determined that miR-28-5p targets Cyclin D1, an important regulator of cell cycle progression, in particular of the G1-S phase transition. Additionally, miR-28-5p directly targets HOXB3, a transcription factor described to be upregulated in colon cancer. On the other hand, miR-28-3p directly regulates the expression of NM23-H1, an anti-metastatic gene.

miR-28 is part of an extensive number of microRNAs deregulated in colorectal cancer. It is essential to understand the effect of the microRNAs in cancer to be able to interfere with the tumor biology and consequently generate therapeutic strategies based on microRNAs as an approach to fight cancer.

Resumo

RNAs não codificantes são uma classe de transcritos de RNA que não codificam proteína. Dentro desta classe encontram-se os microRNAs, pequenos RNAs com 20 nucleótidos de comprimento. Os microRNAs actuam após a transcrição e ligam-se por complementariedade de bases a RNAs mensageiros, nas suas regiões 5´- ou 3´-ultra-conversadas e nas regiões codificantes. Os microRNAs inibem a tradução ou causam a degradação do RNA mensageiro. Além disso, os microRNAs podem também ligar-se a pseudo-genes e estar envolvidos em funções de RNA "decoy". Um único microRNA pode ligar-se a vários RNAs, e um único RNA pode ser o alvo de vários microRNAs. Assim sendo, os microRNAs constituem excelentes reguladores da expressão genética e são elementos chave no controlo das funções celulares.

Os microRNAs estão desregulados em todos os tipos de tumor e podem actuar como supressores tumorais ou como oncogenes, dependendo do tipo de tecido e de célula. Os microRNAs controlam mecanismos biológicos essenciais para as células neoplásicas, incluindo a proliferação, a morte e o ciclo celular, o metabolismo, a hipoxia, a angiogénese, a inflamação, a migração, a invasão, a metastização e a auto-renovação das células estaminais tumorais. Para além da importância dos microRNAs como ferramentas de diagnóstico e prognóstico oncológico, e devido ao seu envolvimento na biologia tumoral, os microRNAs têm sido apresentados como novos alvos terapêuticos. Esta tese analisa o papel dos microRNAs no cancro colorectal. Apesar dos significativos avanços terapêuticos, o cancro colorectal continua a ser um dos tipos tumorais mais diagnosticados e uma frequente causa de morte por cancro. No trabalho aqui apresentado, pretende-se analisar o efeito biológico do miR-28 neste tipo tumoral. Para atingir este objectivo utilizámos ferramentas *in vitro* e *in vivo*.

Geralmente, na via da biogénese dos microRNAs, um microRNA-primário é processado constituindo o microRNA-precursor que, por sua vez, origina um único microRNA maduro. No entanto, em alguns casos, são originados dois microRNAs

maduros. Esta situação ocorre com o precursor do miR-28 que é processado em dois microRNAs maduros designados de miR-28-5p e miR-28-3p. Identificámos, pela primeira vez, ambas as formas do miR-28 maduro como estando desreguladas em cancro colorectal. Contrariamente a outros tipos de tumores/células nas quais o miR-28 está sobreexpresso, no cancro colorectal o miR-28 está subexpresso no tumor quando comparado com os seus níveis de expressão no tecido colorectal normal. No entanto, as duas formas maduras têm funções distintas. Os estudos *in vitro* revelaram que a sobreexpressão do miR-28-5p diminui a proliferação, aumenta a apoptose, impede a progressão do ciclo celular na fase G1, e diminui a migração e a invasão; enquanto que a sobreexpressão do miR-28-3p não tem efeito na proliferação mas aumenta a migração e a invasão. Esta é a primeira descrição do duplo papel do miR-28 em neoplasia colorectal. Visto que as formas maduras do miR-28 são transcritas conjuntamente, analisámos o resultado global da expressão do miR-28 *in vivo*. A injecção, em murganhos, de células neoplásicas colorectais que sobreexpressam miR-28 causou um crescimento mais lento dos tumores, mas provocou um aumento no número de metastases, quando comparámos com o grupo control. Nesta tese, discutimos as estratégias para inibir ou restaurar a função dos microRNAs. O duplo papel do miR-28 tem um vasto impacto no desenho de terapias que recorram a microRNAs, em particular na sobreexpressão dos níveis de microRNAs usando vectores de expressão.

A variação dos níveis de microRNAs tem um impacto tremendo na rede de sinalização intracellular. Observámos que o miR-28-5p tem como alvo a Cyclin D1, um importante regulador da progressão no ciclo celular, em particular na transição G1-S. Além disso, o miR-28-5p tem como alvo directo o HOXB3, um factor de transcrição descrito como estando sobreexpresso no cancro do colon. Por outro lado, o miR-28-3p regula directamente a expressão de NM23-H1, um gene anti-metastático.

O miR-28 faz parte de um extenso número de microRNAs que está desregulado em neoplasias. Compreender o efeito dos microRNAs no cancro é essencial para interferir com a sua biologia e, consequentemente, gerar estratégias terapêuticas utilizando microRNAs na luta contra esta doença devastadora.

Contents

Contents

PAPER I - Almeida MI, Reis RM, Calin GA. MicroRNAs and metastases—the neuroblastoma link. **Cancer biology & therapy**. 2010;9(6):453-4.

PAPER II - Almeida MI, Reis RM, Calin GA. MYC-microRNA-9-metastasis connection in breast cancer. **Cell Research**. 2010;20(6):603-4.

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PAPER V - Almeida MI, Reis RM, Calin GA. MicroRNA history: discovery, recent applications, and next frontiers. **Mutation Research**. 2011;717(1-2):1-8.

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Abbreviations

AMOs - anti-miRNA oligonucleotides

Alg-1 and **Alg**-2 - argonaute (plant)-Like gene (in Caenorhabditis elegans)

APC - adenomatous polyposis coli

BAX - BCL2-associated X protein

BCL2- B-cell CLL/lymphoma 2

BCL6 - B-cell CLL/lymphoma 6

BIM - (synonym: BCL2L11) BCL2-like 11 (apoptosis facilitator)

BNIP2 - BCL2/adenovirus E1B 19kDa interacting protein 2

BP - base pair

BRAF - v-raf murine sarcoma viral oncogene homolog B1

BUB1 - budding uninhibited by benzimidazoles 1 homolog (yeast)

CASP5 - caspase 5, apoptosis-related cysteine peptidase

CCND1 - cyclin D1

CCND2 - cyclin D2

CDC4 - (synonym: FBXW7) F-box and WD repeat domain containing 7, E3 ubiquitin protein ligase

CDC42 - cell division cycle 42 (GTP binding protein, 25kDa)

CDH1- cadherin 1, type 1, E-cadherin (epithelial)

CDK2 - cyclin-dependent kinase 2

CDK4 - cyclin-dependent kinase 4

CDK6 - cyclin-dependent kinase 6

CDS - coding sequences

CEBPA - CCAAT/enhancer binding protein

CIMP - CpG island methylator phenotype

CIN - chromosome instability

CLL - chronic lymphocytic leukemia

CNOT6L - CCR4-NOT transcription complex, subunit 6-like

CpG - adjacent cytosine and guanine dinucleotides

CSC - cancer stem cell

CSDC2 - cold shock domain containing C2, RNA binding

CTNNB - catenin (cadherin-associated protein), beta 1, 88kDa

DGCR8 - DiGeorge syndrome critical region gene 8

DNA - deoxyribonucleic acid

DTL - denticleless E3 ubiquitin protein ligase homolog (Drosophila)

ECM - extracellular matrix

EGF - epithelial growth factor

EGFR - (synonym: HER1) epithelial growth factor receptor

EMT - epithelial-mesenchymal transition

ERK1/2/5 - (Synonym: MAPK3/ MAPK1/ MAPK7) extracellular-signal-regulated kinases 1, 2 and 5 or mitogen-activated protein kinase 3, 1 and 7

E2F3 - E2F transcription factor 3

FAP - familial adenomatous polyposis

FDA - food and drug administration

FOBT - fecal occult blood test

FOXO4 - forkhead box O4

GSK-3β - glycogen synthase kinase 3 β

HBL-1 - HunchBack Like (fly gap gene related)

HCV - hepatitis C virus

HNPCC - hereditary nonpolyposis colorectal cancer syndrome

hnRNP E2 – (synonym: PCBP2) poly(rC) binding protein 2

HOX - homeobox

IARC - International Agency for Research on Cancer

IGF2R - insulin-like growth factor 2 receptor

ITF-2 - (Synonym: TCF4) transcription factor 4

KRAS - v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog

KRAS1P - v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog pseudogene 1

LEF - lymphoid-enhancer factor

LNA - locked nucleic acid

LOH - Loss of heterozygosity

MAML1- mastermind-like 1 (Drosophila)

MEF2C - myocyte enhancer factor 2C

miRNA - microRNA

miR-SP - microRNA sponges

MMP7- matrix metallopeptidase 7 (matrilysin, uterine)

MMR - mismatch repair

MLH1 - mutL homolog 1, colon cancer, nonpolyposis type 2 (E. coli)

MLH3 - mutL homolog 3 (E. coli)

MRE - microRNA responsive element

mRNA - messenger RNA

MSI - microsatellite instability (H- High; L- Low)

MSH2 - mutS homolog 2, colon cancer, nonpolyposis type 1 (E. coli)

MSH3 - mutS homolog 3 (E. coli)

MSH6 - mutS homolog 6 (E. coli)

MSS - microsatellite stable

MuTaME - mutually targeted MRE enrichment

MTHFR - methylenetetrahydrofolate reductase (NAD(P)H)

MYC - v-myc myelocytomatosis viral oncogene homolog

ncRNAs - non-coding RNAs

NK-kB - nuclear factor-kappa B

NIRF - Np95 ICBP90 RING finger

NM23-H1 - (synonym: NME1) NME/NM23 nucleoside diphosphate kinase 1

NRAS - neuroblastoma RAS viral (v-ras) oncogene homolog

NSCLC - non-small cell lung cancer

NUP210 - nucleoporin 210kDa

NT - nucleotide

OCT-4 - (synonym: Pou5f1) POU domain, class 5, transcription factor 1

PACT - (synonym: PRKRA) protein kinase, interferon-inducible double stranded RNA dependent activator

PDCD4 - programmed cell death 4 (neoplastic transformation inhibitor)

PHB - (Synonym: PHABULOSA) homeobox-leucine zipper protein ATHB-14

PIK3CA - phosphoinositide-3-kinase, catalytic, alpha polypeptide

PMS1 - PMS1 postmeiotic segregation increased 1 (S. cerevisiae)

PMS2 - PMS2 postmeiotic segregation increased 2 (S. cerevisiae)

Pre-miRNA - precursor microRNA

Pri-miRNAs - primary microRNA

PTEN - phosphatase and tensin homolog

PTENP1 - phosphatase and tensin homolog pseudogene 1

P21Cip1 - (synonym: WAF1, CDKN1A) cyclin-dependent kinase inhibitor 1A (p21, Cip1)

RDE-1 - RNAi DEfective family member

RHOA - ras homolog family member A

RHOC - ras homolog family member C

RISC - RNA-induced silencing complex

RNA - ribonucleic acid

RNAi - RNA interference

RND3 - Rho family GTPase 3

RT-PCR - Real Time - Polymerase Chain reaction

SERINC1 - serine incorporator 1

shRNAs - short hairpin RNAs

SIRT - sirtuin 1

SIX1 - SIX homeobox 1

SMAD2 and **SMAD4** - SMAD family member 2 and 4

SNP - single-nucleotide polymorphism

SOX2 - SRY-box containing gene 2

TCF - T-cell factor

TGFB1/TGF-β - transforming growth factor, beta 1

TGFβ-RII - (synonym: TGFBR2) transforming growth factor, beta receptor II (70/80kDa)

TGIF2 - TGFB-induced factor homeobox 2

TIAM1 - T-cell lymphoma invasion and metastasis 1

TNM - Tumor-lymph Nodes-Metatasis classification of malignant tumors

TRBP - (synonym: TARBP2) human immunodeficiency virus (HIV)-1 transactivating response (TAR) RNA-binding protein

UTR - untranslated region

VAPA - VAMP (vesicle-associated membrane protein)-associated protein A

VEGFA - (synonym: VEGF) vascular endothelial growth factor A

WT1 - wilms tumor 1

XRCC1 - x-ray repair complementing defective repair in Chinese hamster cells 1

ZAP-70 - zeta-chain (TCR) associated protein kinase 70kDa

ZEB1 - zinc finger E-box binding homeobox 1

ZEB2 - zinc finger E-box binding homeobox 2

5-FU - 5-fluorouracil

Objectives and Thesis Layout

Objectives and Thesis Layout

microRNAs are key players in regulation of biological processes, by controlling the expression of protein-coding messenger RNAs and non-protein-coding transcripts. In cancer, microRNAs control proliferation, apoptosis, cell-cycle, cancer stem cells self-renewal, metastasis and angiogenesis. In addition, microRNAs can be vehicles of resistance to cancer treatment and are considered potential therapeutic targets.

The **general aim** of the work reported in this thesis is to understand how deregulated microRNAs can contribute to tumorigenesis, with a very special emphasis in colorectal cancer, by investigating the role of strand-specific microRNAs (-5p, -3p), originated from the same precursor-microRNA, in this tumor type. In the quest to accomplish this objective, this thesis is organized in individual chapters, for a better elucidation of the work performed, as presented below.

Chapter 1 presents a general introduction on the state of the art of microRNAs and colorectal cancer. It is divided in two sections. The first section focuses on microRNAs (sub-chapter 1.1). It aims to summarize the main reports on the topic and it includes a historical perspective on how microRNAs were discovered, a description of the microRNA´s biogenesis pathway and of the microRNA´s functions, a highlight on the most significant reports associating microRNAs to cancer, an explanation of the utility of microRNAs as molecular biomarkers for diagnosis, prognosis and prediction of therapeutic response in cancer, and finally, a report on the most successful strategies for the use of microRNAs to treat diseases. The second section focuses on colorectal cancer and intends to prepare the reader for a general overview on this disease (sub-chapter 1.2). Regarding colorectal cancer, it stresses the incidence and risk factors, screening methods and diagnostic tools, tumor origin and classical classification, molecular classification and disrupted genetic pathways, and finally some of the currently treatment strategies used. Also in this section, connections between colorectal cancer and microRNAs are established.

Chapter 2 presents the study that explored the biological roles of miR-28 in colorectal cancer. It results from the investigation on the strand-specific miR-28-5p and miR-28-3p effects

in vitro and the overall effect of the miR-28 *in vivo*. This work comprises three specific aims: 1) to analyze the miR-28 expression levels in colorectal cancer and normal colorectal tissue 2) to understand how miR-28 contributes to tumorigenesis in colorectal cancer; 3) to identify miR-28 targets in colorectal cancer cells.

Chapter 3 attempts to discuss and bring together the findings accomplished on chapter 2 with the milieu of other relevant published data. A general concluding remarks section is also included and it emphasises the major findings of the work presented throughout the thesis.

Finally, an **Appendix section** was included and it encloses the additional related studies performed during the PhD time course.

1. General Introduction

Part of the introduction presented throughout this chapter was:

(i) Published as review articles or commentaries in international peer reviewed journals:

Appendix section: PAPER III - Almeida MI, Reis RM, Calin GA. BRCA1, microRNAs and cancer predisposition: challenging the dogma. Cell Cycle. 2011;10(3): 377.

Appendix section: PAPER V - Almeida MI, Reis RM, Calin GA. MicroRNA history: discovery, recent applications, and next frontiers. Mutation Research. 2011;717(1-2): 1-8.

Appendix section: PAPER VII - Spizzo R, Almeida MI, Colombatti A, Calin GA. Long non-coding RNAs and cancer: a new frontier of translational research? Oncogene. 2012. (*in press*)

Appendix section: PAPER VIII - Almeida MI, Reis RM, Calin GA. Decoy activity through microRNAs: the therapeutic implications. Expert Opinion on Biological Therapy. 2012. (*in press*)

1. GENERAL INTRODUCTION

1.1. microRNAs: SMALL NON-CODING RNAS

For decades, the majority of the molecular geneticists focused their work on a protein-centric view that was based on the central dogma that deoxyribonucleic acid (DNA) is transcribed into ribonucleic acid (RNA) which is then traduced into protein. According to this view, the RNA was regarded as a mere intermediate between DNA and proteins [1]. However, the human genome projects came to clarify that the majority of the human DNA does not codify for protein. In fact, humans have approximately the same number of protein coding genes (~20,000) than *Caenorhabditis elegans,* even though humans contain about 10^{14} cells while *Caenorhabditis* elegans contains merely ~1000 cells [2]. Surprisingly, along evolution it is not the extent of protein coding genes that increases, but the extent of non-protein coding DNA, which reaches 98,8% in humans. This suggests that higher developmental complexity may rely on the non-protein coding sequences [2]. Additionally, while only 2% of the mammalians genome codifies for protein, the majority of the genome is transcribed, composing the class of non-coding RNAs (ncRNAs) [2]. The use of massively parallel sequencing platforms of the "next-generation sequencing", with the RNA sequencing (RNA-seq) application, revealed thousands of transcripts and opened the door to a comprehensive study of the non-protein-coding transcriptome [3, 4]. Although the majority of these transcripts have not been experimentally tested yet, what was previously assumed to be "junk" DNA because it was poorly understood and because of established dogmas, is now regarded as extremely important regulatory elements that may hold the key for the evolutionary complexity and for the control of the biological mechanisms [2, 5, 6]. Therefore, it is not surprising that, when disrupted, ncRNAs interfere with normal cellular function and contribute to disease pathogenesis [5, 7].

Presently, several classes of ncRNAs have been established and their functions are being revealed. ncRNAs can be classified in main classes: long-ncRNAs (*e.g.*, long intergenic ncRNAs, antisense ncRNAs, transcribed-ultraconserved regions, promoter

upstream transcripts); structural RNAs (*e.g.*, transfer RNAs, small nuclear RNAs, spliceosomal RNAs); and small-RNAs (*e.g.*, microRNAs [miRNAs], piwi-interacting RNAs, small nucleolar RNAs, promoter-associated small RNAs, transcription initiation RNAs). For a complete revision on long-ncRNAs, including it definition, classification, function and involvement in cancer please refer to Appendix section (PAPER VII). This thesis will focus on the subclass of small-ncRNAs called miRNAs that are among all ncRNAs probably one of the most explored in disease so far.

miRNAs are approximately 20 nucleotides (nt) in length and regulate gene expression posttranscriptionally by binding to 3´untranslated regions (UTR), coding sequences (CDS) or 5´UTR of target messenger RNAs (mRNAs), and lead to inhibition of translation or mRNA degradation [8-10]. miRNAs control the expression of genes involved in several biologic processes, including proliferation, apoptosis, differentiation, and metastasis [8-10]. Two decades ago, both the existence and the importance of miRNAs were completely unknown. Until then, the scientific community focused on genes that codify for protein. The classical dogma that DNA is transcribed into RNA, which then is translated into protein, pushed aside the study of all the non-protein-coding sequences. Only in 1993 did the importance of miRNAs begin to be revealed [11, 12].

Currently, thousands of miRNAs have been identified in humans and other species. miRNA online sequences repositories, such as the miRBase database (http://www.mirbase.org/), are available [13–15]. The miRBase release18 contains 18226 entries representing hairpin precursor miRNAs (pre-miRNAs), expressing 21643 mature miRNA products, in 168 species. Furthermore, current tools and softwares developed for miRNA target prediction facilitate studies about the miRNAs functional network [16, 17]. Currently, in PubMed (http://www.ncbi.nlm.nih.gov/pubmed/), the number of publications has been exponentially growing [18], accounting for more than 15,000 publications (PubMed entries that reference the term ‘microRNA’). The most significant publications on the miRNA field since 1993 are represented in Figure 1 [19].

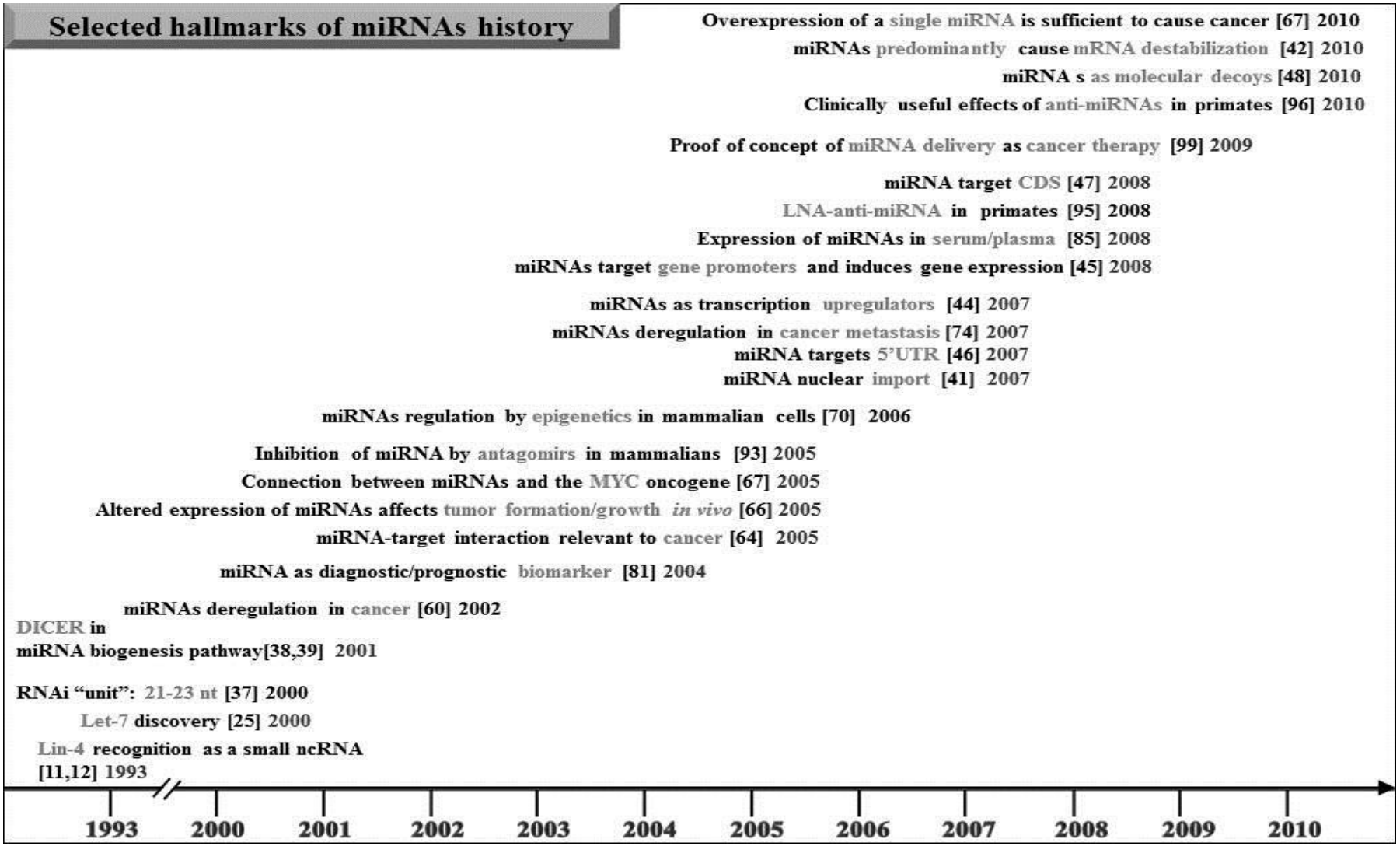


Figure 1. Historical perspective on the evolution of our knowledge about microRNAs (Appendix section, PAPER V, [19])

1.1.1 Historical Perspective on microRNAs

1.1.1.1 Discovery of the First microRNA: lin-4

Lin-4 was the first miRNA to be discovered, in 1993, by the joint efforts of Ambros's and Ruvkun's laboratories [11, 12]. In the nematode *Caenorhabditis elegans*, heterochronic genes control the temporal development pattern of all larval stages. One of these genes is lin-4, discovered by the isolation of a null mutation that causes a failure in temporal development [20, 21]. Animals with lin-4 loss-of-function mutations are missing some adult structures, are incapable of laying eggs, and reiterate early development programs at inappropriate late larval stages. Lin-4 activity is required for the transition from the L1 to L2 stage of larval development [21, 22]. In 1987, Ferguson *et al.*, at Horvitz's laboratory, found that a suppressor mutation in the gene lin-14 was able to revert the null-lin-4 mutation phenotype [23]. In fact, null mutations in lin-14 gene caused an exactly opposite phenotype of the null-lin-4 mutations [22, 23]. This interesting opposite phenotype between defects in lin-4 and lin-14 genes indicated that lin-4 could negatively regulate lin-14 [22]. In 1989, Ambros worked with Ruvkun, at Horvitz's laboratory, to clone the lin-14 gene [22, 24]. At this time, the two colleagues followed two independent research careers, with Ambros focusing on the lin-4 gene and Ruvkun on the lin-14 gene. Ambros, together with Lee and Feinbaum, found that a 700-base pair fragment could contain lin-4 gene but could not find the conventional start and stop codons. Even so, they introduced mutations in the putative open reading frame but lin-4 function remained unchanged. Therefore, Ambros concluded that lin-4 did not encode a protein [11, 22]. In addition, they found two very small lin-4 transcripts of only 61 nt and 22 nt in length [11]. On the other hand, Ruvkun and his colleagues Wightman and Ha found that lin-14 was downregulated at a posttranscriptional level and that the lin-14 3´UTR region was sufficient for the temporal regulation [12]. The two groups shared their unpublished data, and in June 1992 Ambros and Ruvkun independently came to the same conclusion: lin-4 transcripts were complementary to a repeated sequence in the 3´UTR of the lin-14 gene [11, 12, 22]. In December 1993, Ambros and Ruvkun independently reported in the same issue of Cell that the small and non-protein-coding transcript lin-4 regulates

lin-14 through its 3´UTR region [11, 12, 22]. A new unexpected cellular regulatory mechanism involving a non-protein-coding transcript had been found!

1.1.1.2 Discovery of a Second microRNA: let-7

Likewise lin-4, let-7 is a heterochronic gene of *Caenorhabditis elegans* and was the second miRNA to be discovered, in 2000, seven years after the finding of the first miRNA. Reinhart *et al*. at Ruvkun's laboratory reported that let-7 was a 21-nt RNA controlling the L4-to-adult transition of larval development [25]. Loss of let-7 activity causes reappearance of larval cell fates during the adult stage of development, while increased let-7 activity causes precocious expression of adult fates [25]. Remarkably, the authors found that the retarded let-7 phenotype could partially suppress lin-41 (a let-7 target) loss-of- function mutations [25]. In fact, let-7 is complementary to two closely spaced sites in lin-41 3´UTR. Deletion of the lin-41 3´UTR and let-7 mutations abolish lin-41 downregulation, showing that both partners are necessary for this mechanism [26, 27]. Let-7 controls late temporal transitions during development across animal phylogeny. Unlike lin-4, the let-7 sequence is conserved across species from flies to humans [28], a fact that had a major effect on the study of miRNAs in other organisms [29, 30]. Let-7 RNA was detected in vertebrate, ascidian, hemichordate, mollusk, annelid, and arthropod but not in RNAs from plant and unicellular organisms [28, 30]. In humans, it was detected at different expression levels in the majority of the tissues, including brain, heart, kidney, liver, lung, trachea, colon, small intestine, spleen, stomach, and thymus [28]. The let-7 miRNA family refers to miRNAs that share complete sequence identity with let-7 at the 5´ends, termed seed regions, and therefore can regulate the same targets [29]. The let-7 family within humans comprises 12 miRNAs. Some members of the let-7 family identified and functionally analyzed in *Caenorhabditis elegans* include miR-48, miR-84, and miR-241 [31–33]. These miRNAs act redundantly to control the L2-to-L3 transition, by repressing hbl-1 [33]. Functional cooperation among miRNA family members continues to be studied [34]. The discovery that let-7 is conserved across species triggered a revolution in the research of the new class of small ncRNAs, called miRNAs.

1.1.2 Biogenesis Pathway

miRNAs are encoded in the genome as long primary transcripts (named pri-miRNAs) that contain a cap structure at the 5´end and are poly-adenylated at the 3´end. Pri-miRNAs are processed by the cellular RNaseII enconucleaseIII Drosha, together with DGCR8/Pasha protein, into a structure of 60–110 nt, called precursor-miRNA (pre-miRNA), which is then exported from the nucleus to the cytoplasm by an Exportin-5-dependent mechanism. In the cytoplasm, the pre-miRNA is cleft by the RNaseIII enzyme Dicer, together with TRBP/PACT proteins, producing a short imperfect double-stranded miRNA duplex. This duplex is then unwound by an helicase into a mature miRNA, approximately 20 nt in length. Mature miRNA is then incorporated into a multicomponent complex known as RNA-induced silencing complex (RISC), which core components are Argonaute family protein members [35, 36] (Figure 2).

In a historical perspective, the knowledge acquired from RNAi was determinant for understanding miRNAs processing and activity. In 2000, Zamore *et al.* studied the RNAi process and found that double-stranded RNA fragments of 21–23 nt were targeting the mRNA cleavage [37]. The functional unit of RNA interference (RNAi) was therefore the same size as miRNAs. In 2001, two reports were crucial for elucidation of the miRNA biogenesis mechanism, as both reports suggested an involvement of RNAi pathway components in miRNAs maturation [38, 39]. Grishok and colleagues showed that a homologue of *Drosophila* Dicer (dcr-1) and two homologues of rde-1 (alg-1 and alg-2) were essential for lin-4 and let-7 activity in *Caenorhabditis elegans*. Inactivation of these genes caused phenotypes similar to lin-4 and let-7 mutations [38]. Simultaneously, Hutvágner *et al.* found that let-7 pre-miRNA is cleaved by Dicer. In fact, when cells were transfected with siRNA duplex corresponding to human Dicer enzyme, pre-let-7 accumulated in the cells [39]. Taken together, these two studies corroborated the intersection between RNAi and miRNA pathway and opened the door for understanding the formation of mature miRNAs.

In the miRNA biogenesis pathway, Drosha and Dicer are spatially separated, being localized in the nucleus and the cytoplasm, respectively (Figure 2). In 2004, Lund

et al. reported that Exportin-5 was the key player mediating the efficient nuclear export of the short miRNA precursors [40]. However, some miRNAs such as miR-29b are predominantly located in the nucleus [41]. In 2007, Hwang *et al.* found that some miRNAs contain additional sequence elements that control their subcellular localization. In fact, miR-29b possesses a hexanucleotide terminal motif that directs its import into the nucleus. These authors showed that, despite the small size of miRNAs, they can contain additional cis-acting regulatory motifs that might influence their posttranscriptional behavior, and they concluded that miRNAs with common 5´ends, predicted to regulate the same targets, might have distinct functions [41].

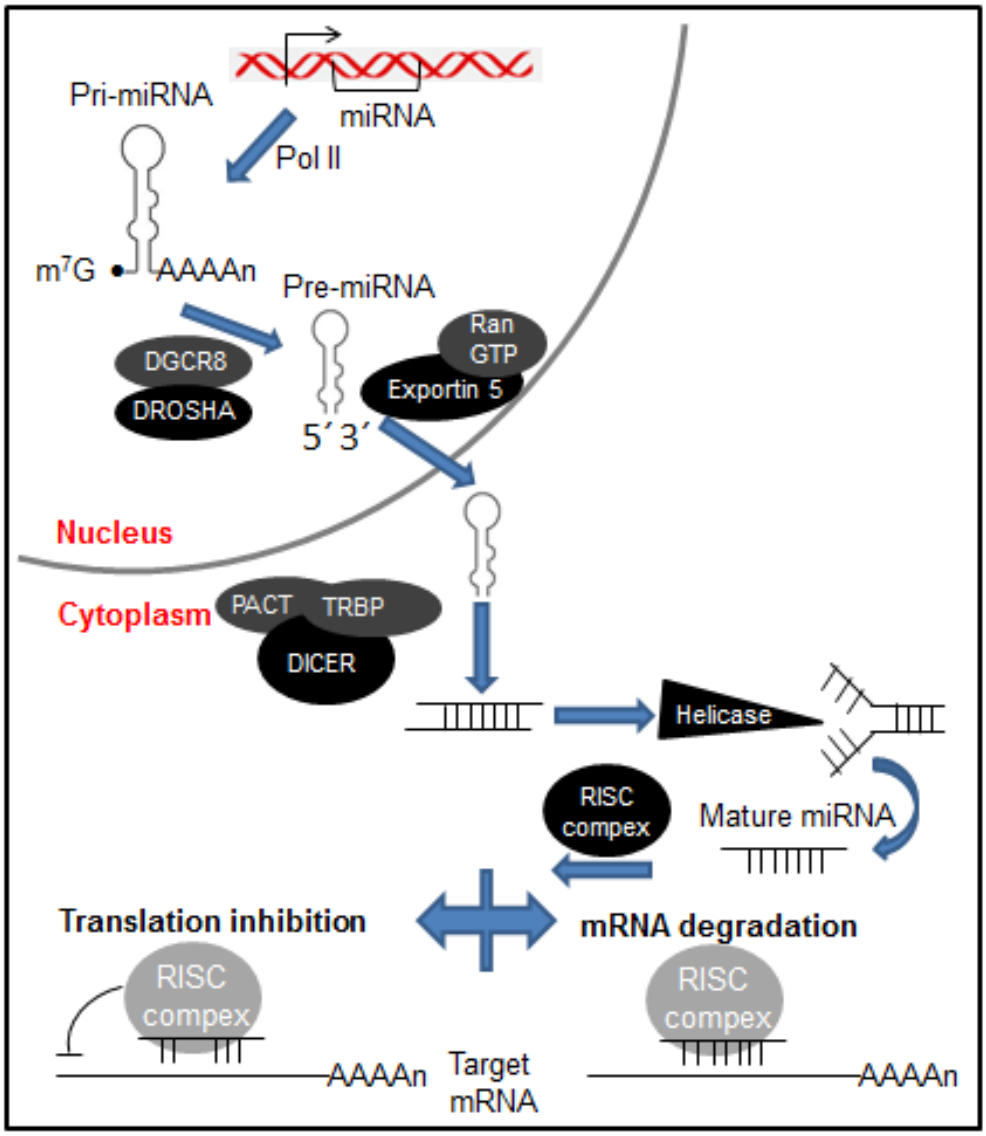


Figure 2. miRNA biogenesis pathway. A primary-miRNA (pri-miRNAs) is transcribed from the genome by a RNA polymerase (Pol) II. Pri-miRNAs harbor a 5´methylated cap

and a 3´poly(A) tail, and forms a hairpin-loop structure. It is then processed by DROSHA/DiGeorge syndrome critical region gene 8 (DGCR8) into a precursor-miRNA (pre-miRNA), which is exported from the nucleus to the cytoplasm via GTP-dependent Exportin 5. The hairpin pre-miRNA is processed by Dicer together with PACT and Dicer-TAR RNA binding protein (TRBP) into a mature miRNA duplex. This duplex is unwound by a helicase. The mature miRNA is incorporated into the RNA-induced silencing complex (RISC). Depending upon the degree of complementarity between the mRNA and the miRNA sequence, miRNAs can inhibit mRNA translation or cause mRNA degradation.

1.1.3 Function

miRNAs' main function is to inhibit protein synthesis of protein-coding genes, either by inhibition of translation or mRNA degradation (Figure 2). However, the relative contribution of each mechanism to repression was unknown until recently. In an elegant study, Guo *et al.* used ribosome profiling to measure the overall effects on protein production and simultaneously measured effects on mRNA levels. They concluded that inhibition of translation (no changes in mRNA levels of miRNA targets) had a modest influence on repressing protein levels, whereas mRNA destabilization was the predominant miRNAs mechanism of action to decrease their targets levels [42]. In addition to mRNAs repression, miRNAs have been also reported to activate translation of targeted mRNAs [43, 44]. Vasudevan *et al.* were the first to clearly demonstrate that, in some instances, miRNAs can work as translational activators. TNFα AU-rich elements recruited miR-369-3 to mediate translation upregulation, exclusively under serum starvation conditions. In addition, upon cell cycle arrest, let-7 and the synthetic miRNA-cxcr4 induced translation, whereas they repressed translation in proliferating cells. Therefore, miRNAs can switch between translation repression and activation in coordination with the cell cycle [44]. In 2008, Place *et al.* provided new evidence that miRNAs can induce gene expression and were the first to show that miRNAs can target gene promoters. These authors showed that miR-373 targets the promoter of E-cadherin and CSDC2 and induced their expression [45]. For a

long time, studies on miRNA-target interaction were confined to 3´UTR of mRNAs, probably because the first studies on miRNAs focused on this region. In 2007, Lytle *et al.* were the first to suggest that miRNAs could associate to any position of target mRNAs and demonstrated that mRNA targets were efficiently repressed by miRNA-binding sites in 5´UTR [46]. In 2008, Tay *et al.* reported that binding sites in coding sequences are abundant and experimentally showed that mouse Nanog, Oct4, and Sox2 have miRNA-binding sites in their coding sequences. miRNAs targeting these mRNAs modulate embryonic stem cell differentiation [47].

Recently, another function for miRNAs was found: the decoy activity. In 2010, Eiring *et al.* reported a remarkable finding for our understanding of how miRNAs function. These authors found that, in addition to miRNAs gene silencing activity through base pairing with mRNA targets, miRNAs also have decoy activity that interferes with the function of regulatory proteins [48]. hnRNP E2 is a member of the family of RNA-binding proteins whose members are involved in mRNA processing, nucleocytoplasmic export, and translation of mRNAs [49]. In cells in which the *BCR/ABL* oncogene is expressed, causing an arrest of differentiation in myeloid blast crisis chronic myelogenous leukemia, there is an increase of hnRNP E2 protein [50]. hnRNP E2 interacts with the transcription factor *CEBPA*, inhibiting the translation of mRNA. Using RNA electrophoretic mobility shift assays, UV crosslinking, and RNA immunoprecipitation assays, the authors found that miR-328 that is downregulated in a BCR/ABL-dependent manner competes with *CEBPA* mRNA for the hnRNP E2 binding site [48]. They also proved that restoration of miR-328 expression interferes with hnRNP E2 function of translation inhibition by preventing *CEBPA*::hnRNPE2 binding and consequently restores, *in vivo* and *in vitro*, *CEBPA* mRNA translation [48]. Besides the decoy activity, miR-328 also functions in the canonical way by suppressing translation of mRNA encoding the PIM1 protein kinase through base pairing interaction [48].

Later the same year, Poliseno *et al.* reported in *Nature* the intriguing discovery that pseudogenes could function as a decoy for miRNAs' effects on corresponding protein-coding genes [51]. The authors used as a model the well-known tumor suppressor *PTEN* and its pseudogene *PTENP1*, which has a high sequence homology with part of the *PTEN* 3´UTR [51]. The authors proved that *PTENP1* is targeted by some

of the miRNAs that target also *PTEN*, including miR-19b and miR-20a. Through a miRNA-dependent mechanism, overexpression of *PTENP1* 3´UTR resulted in the derepression of *PTEN* (and consequently proved that *PTENP1* has a role as a tumor suppressor), and expression of *PTEN* 3´UTR resulted in the derepression of *PTENP1* [51]. In addition, the authors showed that the same decoy mechanism is present when analyzing other genes and their related pseudogenes, such as the *KRAS* gene and its pseudogene *KRAS1P* [51]. This new concept was further developed one year later, when the same group showed that not only non-coding genes can compete for miRNAs binding sites, but also protein-coding transcripts can compete with one another [52]. Transcripts that have the same miRNA binding sites (or miRNA response elements [MREs]) are called "competing endogenous RNAs" (ceRNAs) [53] and may act as natural miRNA sponges. The authors used bioinformatics (MRE enrichment—MuTaME—analysis) and biological approaches to validate ceRNA for *PTEN* [52]. Some of these mRNAs are *SERINC1*, *VAPA*, and *CNOT6L*, whose expression in human prostate cancer and glioblastoma samples was significantly different between PTEN-high and PTEN-low groups [52]. In addition, silencing of these ceRNAs resulted in a decrease in luciferase activity when cells were co-transfected with a luciferase vector containing the *PTEN* 3´UTR. The authors further proved that this correlation was dependent on the miRNAs, since regulation of *PTEN* expression by *SERINC1*, *VAPA*, and *CNOT6L* ceRNAs vanished in the cells with a defect in the miRNA processing machinery [52].

In the same issue of *Cell*, three articles reported "out-of-the-box" discoveries about coding transcripts and competing endogenous RNAs. Sumazin *et al.* used a multivariate analysis method, named Hermes, to combine gene expression data with miRNA profiles in glioblastoma and found 7,000 genes whose transcripts were involved in sponge regulatory interactions (modulator and sponges share miRNAs binding sites) and 148 genes that were involved in nonsponge regulatory interactions (modulator and sponges that do not necessarily share miRNA binding sites) [54]. Moreover, the authors focused on the *PTEN* transcript to perform validation studies for *PTEN* miRNA-mediated decoy in glioblastoma cell lines [54].

In another study, Karreth *et al.* identified *PTEN* ceRNAs in a mouse model of melanoma with use of the sleeping beauty transposon system [55]. The authors

further validated *ZEB2* as a ceRNA decoy for *PTEN* by demonstrating that ZEB2 depletion downregulates PTEN and that this reduction was dependent on the 3′UTR (ZEB depletion suppressed luciferase activity of *PTEN* 3′UTR reporter) and on miRNAs (ZEB2 depletion does not reduce PTEN expression in cells with a defect in the miRNA processing machinery) [55]. Finally, Cesana *et al.* reported that a long noncoding RNA, *linc-MD1*, acts as a ceRNA for *MAML1* and *MEF2C* mRNAs, two transcription factors that regulate muscle-specific genes, by interacting with miR-133 and miR-135, thereby regulating muscle differentiation [56]. All of the above-mentioned studies were crucial to a new understanding of the importance of miRNAs in mediating mRNA decoys, and their significance has been highlighted in several articles. For example, McCarthy [57] stated that a subtle reduction in few mRNA could cause widespread effects. Rigoutsos and Furnari [58] indicated that the relative amount of mRNAs and corresponding ceRNAs should be considered and they introduced the intriguing hypothesis that the decoy mechanism may also occur in cases in which PTEN levels are reduced without mutation, such as in Cowden disease and Bannayan-Zonana syndrome. In addition, Swami [59] questioned how this decoy applied in noncancer-related genes.

In conclusion, the canonical sense of miRNAs function is the gene silencing activity through base pairing with mRNA targets. Additionally, miRNA play a role in decoy activities, which introduces another level of complexity on the miRNAs gene regulatory effects.

1.2.4 microRNAs in Disease

1.1.4.1 microRNAs Involvement in Cancer

The first report suggesting a role of miRNAs in cancer was published in 2002 [60]. miR-15 and miR-16 were found to be located at chromosome 13q14, a region frequently deleted in chronic lymphocytic leukemia (CLL). Calin *et al.* discovered that both genes were deleted or downregulated in greater than 60% of B-cell human CLL, indicating that these genes behave as tumor suppressors in CLL [60]. Consequently, the same group found that a significant percentage of miRNAs is located at fragile sites

and in regions altered in cancers, including regions of amplification or loss of heterozygosity or breakpoints, suggesting that miRNAs have a relevant role in human cancer pathogenesis [61]. Oligonucleotide miRNA microarrays and, more recently, deep sequencing (next generation sequencing) have permitted the analysis of the entire known miRNAome. In addition, other methods such as bead-flow cytometry, quantitative real-time polymerase chain reaction, and high-throughput array-based Klenow enzyme assay have been used to assess miRNA expression in tumors and other diseases. To date, altered miRNA expression had been reported in almost all types of cancer [62]. In 2005, the first reports addressing the biological function of miRNAs in cancer were published. miRNAs can act as oncogenes (oncomirs) or tumor suppressors and are involved in a variety of pathways deregulated in cancer [63]. In March 2005, Johnson *et al.* reported the first miR-target interaction with relevance to cancer. The authors demonstrated that in *Caenorhabditis elegans* let-7 targets let-60, encoding the *Caenorhabditis elegans* ortholog of human oncogene RAS. In addition, they showed that human RAS expression is regulated by let-7 in cell culture. Accordingly, let-7 expression is decreased in lung cancer compared with normal tissue, and it correlates with the increased RAS protein levels detected in lung tumor samples [64]. Cimmino *et al.* reported that miR-15 and miR-16, the first two miRNAs associated with cancer, play a role in apoptosis regulation by targeting the anti-apoptotic *BCL2* mRNA [65]. Also in 2005, He *et al.* studied for the first time the contribution of miRNAs to tumor development *in vivo*. By overexpressing the miR-17-22 cluster, which is upregulated in human lymphoma, they were able to accelerate lymphomagenesis in a mouse B-cell lymphoma model carrying c-myc oncogene [66]. Furthermore, O'Donnell *et al.* reported for the first time that a transcription factor, specifically MYC, modulated the expression of the same cluster of miRNAs and consequently the E2F1 expression [67]. From 2005 until the present, thousands of scientific communications have reported on the role of miRNAs in tumors, as well as the regulation of miRNAs by other transcription factors such as TP53 (for a detailed review see [68]). In 2010, Medina *et al.* reported results in mice that conditionally overexpressed miR-21 (without other predisposing genetic backgrounds), clearly demonstrating that overexpression of a single miRNA, specifically miR-21, was sufficient to cause tumor development. In addition, these authors proved that tumor volume and survival were dependent on

miR-21 overexpression and that the tumors regressed when miR-21 was inactivated, proving for the first time an oncogenic miRNA addiction for tumor cells [69].

miRNAs deregulation can be caused by several mechanisms including deletion, amplification, mutation, or dysregulation of transcription factors that target specific miRNAs. In addition, miRNAs can be controlled by epigenetic mechanisms. In 2006, Saito *et al.* were the first to demonstrate that miRNAs expression could be controlled by the two major epigenetic mechanisms: DNA methylation and histone modifications [70, 71]. When Saito *et al.* simultaneously treated a human bladder carcinoma cell line with 5-aza-2- deoxycytidine (a DNA methylation inhibitor) and 4-phenylbutyric acid (a histone deacetylase inhibitor), 17 of 313 human miRNAs were found to be upregulated. In particular, miR-127 was upregulated after treatment with these drugs and expression of one of its targets, BCL6, was suppressed [70]. Accordingly, miR-127 expression was downregulated in primary human bladder and prostate tumors compared with normal tissue [70]. Therefore, these authors concluded that in cancer tissue miR-127 is subject to epigenetic silencing [70]. This finding opened the field of epigenetics to miRNAs regulation [71]. Interestingly, a bidirectional connection between epigenetics and miRNAs has been established. On the one hand, epigenetic mechanisms control miRNAs; on the other hand, miRNAs can target essential epigenetic key players. One of the first reports suggesting methylation could be controlled by miRNAs was published in 2004; Bao *et al.* suggested that miR-165/166 are important for methylation of PHB gene in *Arabidopsis* [71, 72].

In cancer patients, metastasis is the principal cause of death. The metastatic process involves multiple steps: cell motility, invasion of adjacent stroma, intravasation, systemic dissemination (though either the blood or lymph), extravasation into the parenchyma of distant tissues, and finally proliferation at a new site, giving rise to a secondary tumor. In this process, miRNAs have a dual role as they can promote or inhibit metastasis [73]. The first finding about miRNAs functioning as metastasis activators was reported by Ma *et al.* [74]. MiR-10b positively regulates cell migration and invasion *in vitro* and is capable of initiating tumor invasion and metastasis *in vivo*. MiR-10b acts by directly targeting HOXD10, which is a transcriptional repressor of RHOC, a key player in metastasis. Concordantly, miR-10b

expression is elevated in about 50% of metastatic breast tumors compared with metastasis-free tumors or normal breast tissues [74]. Three years later, the same group found that miR-9 directly targeted E-cadherin (encoded by *CDH1*), a transmembrane protein whose primary function is to mediate cell adhesion [75]. Loss of E-cadherin expression is one of the hallmarks of epithelial-mesenchymal transition (EMT) process [76]. miR-9 upregulation in breast cancer cells suppresses E-cadherin that consequently loses its capacity to sequester β-catenin and potentiates the Wnt signaling [75]. Study by Ma *et al.* is exciting because the authors demonstrate the contribution of miR-9 not only for EMT but also for induction of angiogenesis that is essential for metastasis to develop [75]. VEGFA is a key pro-angiogenic protein and a target of β-catenin [77]. Ma *et al*. show that miR-9 overexpression upregulates VEGFA and that it correlates with E-cadherin expression (for a complete description, refer to Appendix section) [75]. Along the same line, Huang *et al.* found that human miR-373 and miR-520c stimulated cancer cell migration and invasion *in vitro* and *in vivo* [78]. In contrast, miRNAs can prevent tumor metastasis. Tavazoie *et al.* published the initial study of miRNAs as metastasis suppressors [79]. In breast cancer, patients with low expression levels of miR-335, miR-126, and miR-206 had a shorter median time to metastatic relapse. Restoration of their expression in breast cancer cell lines decreased the number of metastases in inoculated mice. These miRNAs have distinct mechanisms for metastasis suppression: restoration of miR-126 expression significantly suppressed overall tumor growth, whereas restoration of miR-335 or miR-206 levels altered cell morphology, possibly causing a decrease in cell motility [79]. It was therefore not surprising that Lujambio *et al.* reported in 2008 that DNA methylation-associated silencing of tumor suppressor miRNAs contributed to the development of human cancer metastasis and that the reintroduction of miR-148a and miR-34b/c in cancer cells with epigenetic inactivation inhibited their motility, reduced tumor growth, and inhibited metastasis formation in xenograft models, with an associated downregulation of the miRNA oncogenic target genes, such as C-MYC, E2F3, CDK6, and TGIF2 [80].

1.1.5 microRNAs as Molecular Biomarkers

In a clinical context, miRNAs can be extremely useful in disease diagnosis and prognosis and in prediction of therapeutic response. In 2004, Takamizawa and coworkers were the first to pinpoint the prognostic value of miRNAs by showing that let-7 expression was reduced in lung cancers and that lung cancer patients with low let-7 expression levels have a significantly shorter survival after potentially curative resection [81]. In 2005, Calin *et al.* reported the first study showing the diagnostic/prognostic importance of miRNAs at the genome-wide level [82]. These authors found that miRNA expression profiles could be used to distinguish normal B cells from malignant B cells in patients with CLL. In fact, a unique miRNA expression signature is associated with prognostic factors such as ZAP-70 expression (predictor of early disease progression) and mutational status of IgVh. In addition, these authors found nine miRNAs that were differently expressed between patients with a short interval from diagnosis to initial therapy and patients with a significantly longer interval. Furthermore, this study also highlighted the fact that one mechanism of miRNA deregulation is mutation: a germline mutation in the precursor of miR-16-1-miR15a caused low levels of miRNA expression both *in vitro* and *in vivo* [82]. Currently, the clinical utility of miRNAs as diagnostic/prognostic biomarkers has been demonstrated in several types of cancer by numerous studies using tumor samples removed during surgery or biopsies [63]. For non-malignant diseases, however, larger and more studies should be conducted.

1.1.5.1 microRNAs as Biomarkers in Plasma or Serum

Current techniques for cancer diagnosis commonly involve a biopsy of the cancer tissue. Because this technique is invasive and unpleasant for patients, some studies have been focused on the search for biomarkers in human fluids such as plasma/serum, urine, or saliva. Blood samples from patients are usually readily available and many biological molecules, as circulating nucleic acids, can be found in blood serum/plasma, including miRNAs [83, 84]. These small non-coding RNAs in the blood are incorporated into microparticles and exosomes (50- to 90-nm membrane

vesicles) that prevent their degradation, conferring an advantage to the use of miRNAs as markers in serum [83]. In addition, detection of miRNAs in serum is easy owing to highly sensitive PCR detection methods, the lack of post-processing modifications of miRNAs, and simple methods of miRNAs extraction from serum [83]. The first report addressing the utility of miRNAs as diagnostic tools in biological fluids was published in 2008 by Chim *et al.* in a study that detected placental miRNAs in the maternal plasma [85]. In the same year, Lawrie *et al.* [86], by comparing serum from patients with diffuse large B-cell lymphoma and healthy controls, found that miR-155, miR-210, and miR-21 levels were significantly upregulated in patients. Interestingly, these miRNAs have been shown to be deregulated in tumors. Moreover, high expression of miR-21 in patients serum was correlated with improved relapse-free survival times [86]. To date, miRNAs deregulation in serum of cancer patients have been described for several cancers, including leukemia, lymphoma, and gastric, colorectal, lung, oral and squamous cell, breast, ovarian, prostate, pancreatic, and hepatocellular cancers [84]. Furthermore, miRNAs plasma levels are also potential diagnostic markers for non-oncologic diseases, eg. rheumatoid arthritis or osteoarthritis, as differences in miRNA plasma levels between patients with these disease and healthy control groups were reported [87, 88]. To translate the evaluation of miRNAs expression in serum into a clinical routine for diagnosis and prognosis, it is still necessary to standardize the methodologies used for these studies, i.e., serum/plasma extraction procedures and storage conditions, housekeeping miRNAs for normalization in serum samples, or the use of the same statistical methods for data analysis [83]. In addition, large studies reporting miRNA levels in plasma and serum with detailed clinical data information, together with normal controls from both sexes and different ages, are still needed [84]. Besides blood, miRNAs can also be found in other body fluids such as saliva and urine [89].

1.1.6 Therapeutic Implications

miRNAs are aberrantly expressed in several diseases; therefore, it is not surprising that these small ncRNAs represent potential therapeutic targets for the

diseases they are functionally associated with. miRNAs that are upregulated in diseases should be targeted using anti-miRNAs, which are antisense oligonucleotides with specific modifications [90]. For instance, antagomirs, a class of anti-miRNAs that is cholesterol-conjugated to facilitate cellular intake and serum protein binding, could be used to block oncomirs in cancer [90, 91]. In 2004, Hutvágner *et al.* successfully copied the phenotype of let-7 loss-of-function mutation by injecting a 2´-O-methyl oligonucleotide complementary to let-7 miRNA into *Caenorhabditis elegans* larvae [92]. In 2005, Krützfeldt *et al.* reported for the first time the use of antagomirs *in vivo* in mammals [93]. Using a mouse model, Krützfeldt and colleagues systemically delivered via intravenous injection antagomirs against miR-16, miR-122, miR-192, and miR-194 that specifically downregulated the corresponding miRNAs. Silencing of miRNAs using antagomirs was long lasting, and miR-16-antagomir effects were detected in multiple tissues, except in the brain, possibly due to the blood-brain barrier [90, 91, 93, 94]. Other approaches to efficiently inhibiting miRNAs *in vivo* include the use of locked nucleic acid (LNA) oligonucleotides or 2´-O-methoxyethyl phosphorothioate modification [91]. Elmén *et al.* evaluated for the first time the effect of an LNA-anti-miRNA in non-human primates, with surprising results. These authors intravenously injected an LNA-anti-miRNA-122 into African green moneys and were able to efficiently silence the mature miR-122. The effect was long-lasting and safe, as neither toxicity associated with LNA nor histopathological changes were detected [95]. Two years later, Lanford *et al.* described the utility of anti-microRNAs for the clinical practice. miR-122 is a liver-expressed miRNA essential for hepatitis C virus (HCV) replication. Using an LNA-anti-miR-122, the authors were able to suppress HCV viremia in chronically HCV-infected chimpanzees. Moreover, this therapy generated a high barrier to resistance, and no side effects were detected [96]. In addition to these direct-inhibitory methodologies, an indirect technology can be used through downregulation of miRNA biogenesis pathway components. Tetracycline-inducible short hairpin RNAs (shRNAs) could be used to downregulate Dicer or Drosha, key components of the miRNA-biogenesis pathway; however, this mechanism should be tightly controlled, as downregulation of this pathway will have an effect on all miRNAs [90, 91]. On the other hand, when miRNAs downregulation promotes disease, as it is the case of some miRNAs downregulated in tumors and that function as tumor

suppressors, a therapeutic approach would be to restore the mature miRNA levels in the proper tissue/cells. In this situation, it should be used synthetic RNA duplexes, resembling siRNA molecules, that will mimics miRNAs duplex and will be recognized by RISC complex [91]. This complex will process it as the endogenous miRNA, by loading the stand with the sequence identical to the mature miRNA. This approach still needs to be evaluated *in vivo* because some challenges, such as stability and delivery strategies, need to be improved [90, 91, 97]. The use of short-hairpins driven by Pol III promoters could be a strategy for re-expression of miRNAs in the cells [97], although the dosage used in this mechanism has to be narrowly controlled, since sustained shRNA high-level expression in livers of adult mice has been shown to be fatal [98]. In 2009, Kota *et al.* demonstrated how useful systemic administration of miRNAs can be for anti-cancer therapy. miR-26a is expressed at low levels in hepatocellular carcinoma but normally expressed in other tissues. These authors used an adeno-associated virus to mediate miR-26a delivery in a mouse model of liver cancer and were able to reduce cancer cell proliferation and induce tumor cell apoptosis, which consequently caused tumor regression. Since only cancer cells present miR-26a downregulation, the delivery was highly specific and did not affect normal tissue, which was tolerant to miR-26a restoration [99].

1.2 COLORECTAL CANCER

1.2.1 Incidence

Cancer is a worldwide leading cause of death, with 12,7 million new cancer cases and 7,6 million cancer deaths in 2008 [100, 101]. According to the last worldwide statistics for cancer occurrence and outcome performed by the International Agency for Research on Cancer (IARC) in 2008 collected data, colorectal cancer is the third most commonly diagnosed tumor type, after lung and breast cancer, accounting for 1,23 million cases (9,7% of total number of cancers) [100, 102, 103]. It is the fourth most common cause of cancer-related death, account for approximately 698,000 deaths. Generally, incidence is higher in men (663,000 cases) than in women (570,000 cases) and the same tendency is observed when mortality is analyzed. Both colorectal

cancer new cases and mortality are higher in developed countries than in developing countries [101] (Figure 3). Developed regions including Australia/New Zealand, Europe and North America have a high number of cases and the highest mortality rates are observed in Central and Eastern Europe [100, 101] (Figure 3).

Among all tumor types, 2008 statistics indicate that in Europe, colorectal cancer is the most common (436,000 cases, 13,6% of the total) and the second most common cause of death from cancer (212,000 deaths, 12,3%) [104]. If we consider the distribution by gender, it is the third and the second major cancer diagnosed in men and women, respectively; and the second leading cause of cancer related death for both men and women [104]. In the United States, data from the National Center for Health Statistics, projected 1,5 million new cases and 569,490 deaths from cancer in 2010, from which 142,570 were estimated new cases of colorectal cancer (third most commonly diagnosed cancer type in both men and women), and 51,370 were estimated deaths from colorectal cancer (third most commonly cancer related death in both in men and women) [105].

In Portugal, the Globocan project reported, for 2008, an incidence of colorectal cancer of 3951 and 3001 cases in men and women, respectively, and 2112 and 1579 deaths in men and women, respectively [106]. The Report on the Implementation of the Council Recommendation on Cancer Screening by the European Commission and IARC, that analyzed the estimates for 2006 in the 27 Member States of the European Union, rank Portugal in the top 10 members with the highest age-standardized rates of mortality of colorectal cancer [107].

Colorectal cancer mortality rates both in Europe and in the US have been declining since last years [104, 105], which reflects more effective screening and prevention by detecting precancerous polyps and tumor at early stages, a reduction in the risk factors and an improvement in cancer treatment [108].

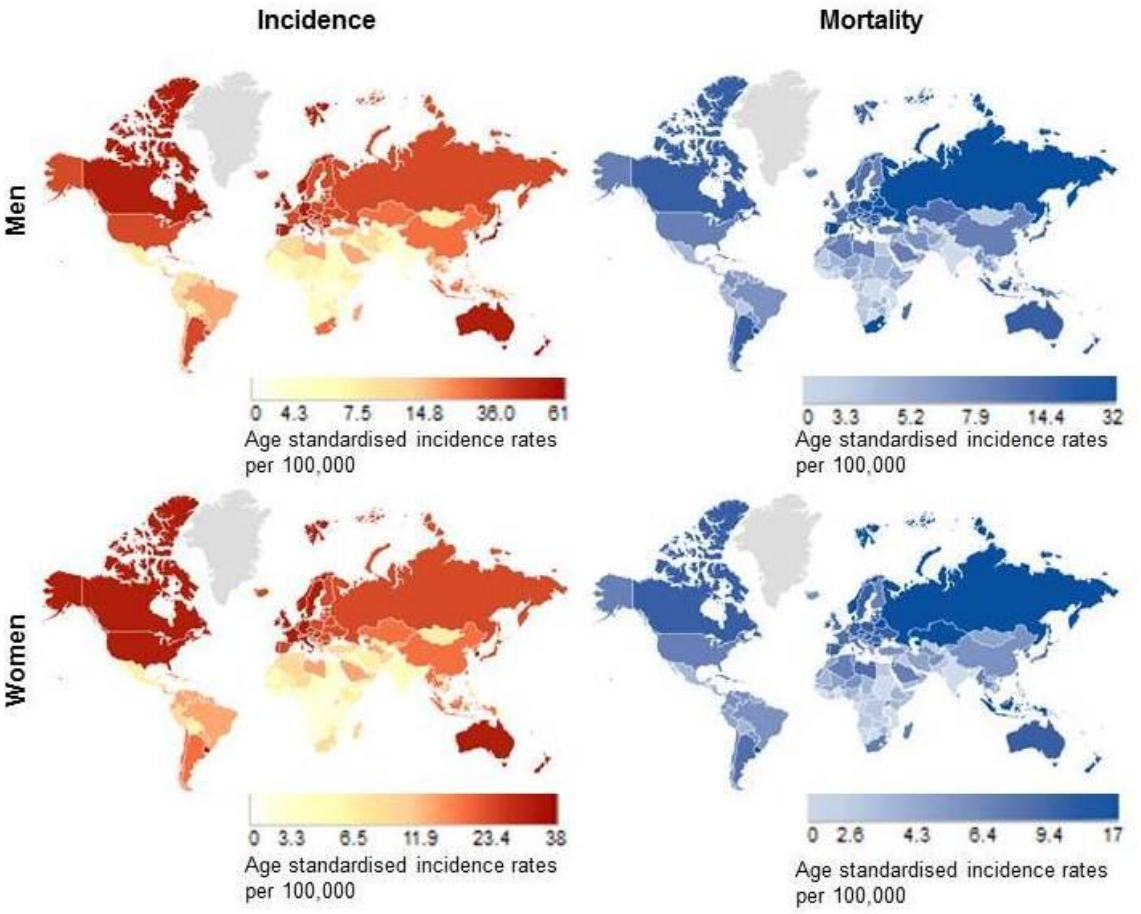
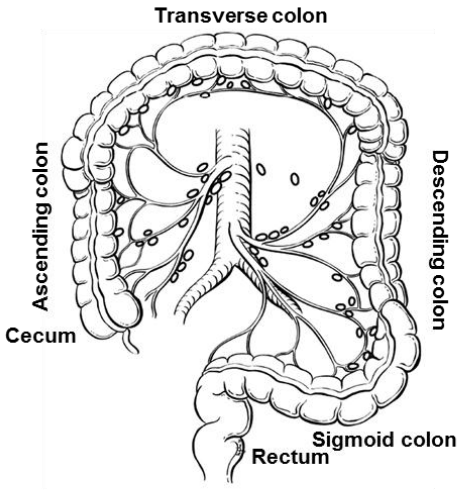


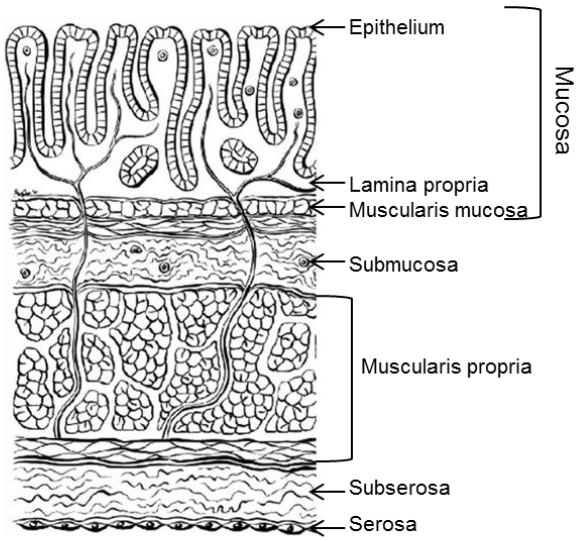
Figure 3. Worldwide incidence and mortality rates by colorectal cancer in men and woman in 2008 (adapted from Globocan 2008 [102, 103]; Ferlay J [106])

1.2.3 Colorectal Cancer Classification and the Tumor-Node-Metastasis Staging System

For over 50 years, the classification system “T (tumor) N (lymph Node) M (metastasis) created by the Union for International Cancer Control and the American Joint Committee on Cancer has been used to classify tumor stages [109, 110]. The system is used to guide treatment strategies, since it allows clinicians to stratify the patients according to primary tumor invasion (Figure 4), lymph nodes metastasis or distant metastasis. It is currently the main tool to provide prognostic information and to determine treatment protocols [111].

Other tumor classifications, based on clinical and pathological features that consider, for example, proximal versus distal, mucinous versus nonmucinous, well-moderate versus poorly differentiated, among others, are extensively used [112]. According to the World Health Organization epithelial colorectal tumors can be classified as adenoma, intraepithelial neoplasia (dysplasia) associated with chronic inflammatory diseases, carcinoma, carcinoid (well differentiated endocrine neoplasm) and mixed carcinoid-adenocarcinoma [113]. The colorectal carcinomas can be subclassified as adenocarcinoma, mucinous carcinomas, signet-ring cell carcinoma, serrated adenocarcinoma, medullary carcinoma, cribriform comedo type adenocarcinoma, micropapillary carcinoma, adenosquamous carcinoma, squamous cell (epidermoid) carcinoma and undifferentiated carcinoma [114, 113]. Due to the tumors heterogeneity, besides clinical and histopathological examination, molecular features are currently used to classify the disease. It additionally allows a better understanding of the pathogenesis, increases knowledge about the therapeutic targets and leds to a personalized therapeutic approach.





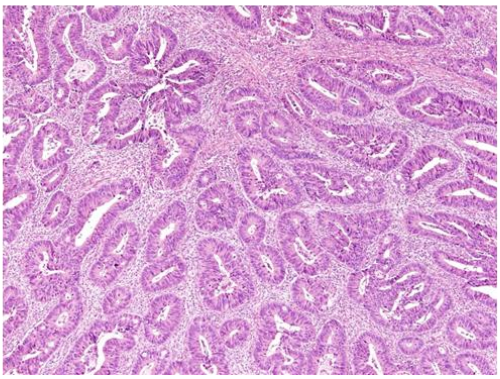


Figure 4. Human colon and rectum. Structure, lymph nodes, and the tissue layers that constitute colorectal wall [Adapted from *Cancer Staging Manual 7th edition* – American Joint Committee on Cancer (http://www.cancerstaging.org/staging/index.html#)]. A histologic representation of colorectal cancer is shown.

1.2.3 Risk Factors in Colorectal Cancer

1.2.3.1 Lifestyle, Environmental and Dietary Colorectal Cancer Risk Factors

Due to the high incidence of colorectal cancer worldwide and in order to be able to prevent and control this disease, epidemiologic studies are essential. Both lifestyle, environmental, dietary, and genetics factors are associated to an increased risk of colorectal cancer. A recent study grouped the carcinogenic agents according to the different tumor types. According to the authors, alcoholic beverages, tobacco smoking, X-radiation and gamma-radiation are carcinogenic agents with sufficient evidences to be associated with colorectal cancer. Limited evidences were found for the association of asbestos and *Schistosoma japonicum* with colorectal cancer [115].

Regarding dietary and nutritional factors, the consumption of red meat has been associated with colorectal cancer by the majority of the studies [116]. The fact that rural Africans have a diet high in fiber and a low incidence of colorectal cancer led to the hypothesis raised in 1971 by Burkitt *et al.* suggesting that a diet rich in fiber could reduce colorectal cancer risk [117, 118]. However, studies are controversial. Aune *et al.* analysed 25 prospective studies and concluded that, although the majority of the studies did not provide enough details, including the subtypes of fiber analyzed and also lacked information about other dietary and lifestyle factors, there was an association between dietary fiber intake (cereal fiber and whole grains) and a linear reduction in the risk of colorectal cancer [117]. The same authors, in a meta-analysis of prospective studies, reported there was a weak inverse association between fruit and vegetable intake and colorectal cancer [119]. Also, an inverse association was observed in studies that assessed vitamin D intake and blood 25-hydroxyvitamin D levels (precursor of vitamin D active form) in relation to the risk of colorectal cancer [120]. In

an attempt to diminish cancer incidence, nutrition guidelines have been published, *e.g.* by the American Cancer Society and the American Institute for Cancer Research [121, 122]. It is estimated that good allowance to dietary guidelines is associated to a 21% reduced risk of colorectal cancer [123].

1.2.3.2 Diseases and Syndromes Associated to Colorectal Cancer

The inflammatory bowel disease presents in two major forms, Crohn's disease and ulcerative colitis, and has been associated to colon cancer. In fact, this connection is one of the paradigms that link inflammation to cancer. Although colorectal cancer does not always develop after a history of inflammatory bowel disease, the association between both dates far backs the late 40´s and early 50´s [124-126]. Due to biases and methodological errors, colorectal cancer risk is difficult to quantify. Depending on the studies, it is estimated that the probability of developing colorectal cancer in patients with ulcerative colitis is 0,4-2% after 10 years, 1,1-8% after 20 years and 3,1-18% after 30 years of the diagnosis. Similar probability of developing colon cancer is found when patients with longstanding Crohn's colitis are considered [127-130]. Moreover, the risk of inflammatory bowel disease patients to develop colorectal cancer is higher in male than in female [131]. Several factors affect colorectal cancer risk in inflammatory bowel disease and these include, the duration and extension of the colitis, family history of colorectal cancer, presence/absence of primary sclerosing cholangitis, severity of histologic cholangitis, age of the inflammatory bowel disease onset, history of dysplasia, surveillance colonoscopy or chemoprevention [132].

Furthermore, there are major cancer susceptibility syndromes that confer an increased risk for colorectal cancer and/or colorectal polyposis, and among these are included, the Lynch syndrome (also known as hereditary nonpolyposis colorectal cancer syndrome - HNPCC), Muir–Torre syndrome, Turcot syndrome, familial adenomatous polyposis (FAP), attenuated familial adenomatous polyposis, *MUTYH*-Associated Polyposis syndrome, hyperplastic polyposis syndrome, Peutz–Jeghers syndrome, juvenile polyposis, and the *PTEN* hamartoma tumor syndrome [133, 134].

Among the non-polyposis syndromes, Lynch syndrome is the most common, accounting for 1-6% of all colon cancers and approximately 10% of the cases before the age of 50 years [133, 134]. This syndrome can also predispose to other tumor types, like endometrium, stomach or ovary, and it is characterized by an autosomal dominant inheritance, an early age of onset and a proximal localization, comparing to sporadic tumors [133, 135].

Among polyposis syndromes, FAP is the most common, accounting for <1% of all colon cancer cases [133]. It is characterized by hundreds of thousands of precancerous colonic polyps [134, 136] and it is transmitted by an autosomal dominant inheritance. It development generally begins, on average, by the age of 16 years, and by the age of 35, 95% of the individuals with this syndrome have polyps [136].

1.2.3.3 Polymorphisms and Colorectal Cancer Risk

Genome-wide association studies of cancer susceptibility have revealed several polymorphisms that confer an increased risk of colorectal cancer [137]. From all known human genetic variants, single-nucleotide polymorphisms (SNPs) are the most frequent and have been extensively explored for disease susceptibility and clinical outcome studies [138]. As between distinct populations, SNP frequencies vary extensively, studies may not be in consensus. Some of the SNPs that have been associated to colorectal cancer risk are rs961253 located in 20p12 (in Asian and European population) [139], rs603965 located in CCND1, G870A polymorphism (especially in Caucasians) [140], rs1800734 located in MLH1 promoter [141], rs12953717 located in SMAD7 gene (in a study where 91% of the population under study was non-Hispanic white and that considered colon cancer only) [142]. Strong candidates for low penetrance susceptibility alleles are HRAS1-variable number tandem repeat, and MTHFR variants [143]. Among all the colorectal cancer associated variations, it is important to highlight the I1307K mutation in the APC gene that is present in 6% of the Ashkenazi Jews [133]. Within this population, but not in Caucasians, this missense mutation causes a substitution of lysine for isoleucine and confers an absolute lifetime risk of around 20% [133, 144].

Genetic variations in miRNAs or in miRNA targets might interfere with miRNA-mRNA interaction by reinforcing, weakening or disrupting it, which consequently may affect expression levels of several proteins involved in cancer genesis and development, such as oncogenes and tumour suppressor genes [145, 137] [Figure 5). SNPs in miRNAs themselves or in miRNA-binding sites may affect individual's risk of cancer [146] (for an example, refer to Appendix section). Regarding SNPs in miRNAs, in the Korean population, Min *et al.* found a significantly increased colorectal cancer risk in individuals with the miR-196a-2-CC (rs11614913) genotype compared with the -TT/CT genotype [147]. In the Chinese population, it was also found that miR-196a-2-CC genotype increased colorectal cancer risk [148, 149], but studies are not unanimous [150]. It is necessary to consider that SNPs´ frequencies may vary between populations. A meta-analysis concluded that miR-196a-2-CC genotype is only associated with Asian but not in Caucasian population [151]. In addition, larger studies are needed. Regarding miRNA-target SNPs in colorectal cancer, the first studies were reported by Landi *et al.* group. The authors performed *in silico* predictions and case–control association studies and determined that variants predicted to be within miRNA-binding sites in CD86 and INSR genes were associated with colorectal cancer risk [152]. Later, the same group reported association of colorectal cancer susceptibility also with alleles of rs709805-*KIAA0182* and rs354476-*NUP210* genes that are within miRNA target sites [153, 154]. Besides *in silico* predictions, same studies also explore the function significance of the miRNA-target variants. Nicoloso *et al.* clearly show through luciferase activity assays and measurement of target protein levels in miRNA post-transfected cells that alleles of the breast cancer associated SNPs rs1982073-TGFB1 and rs1799782-XRCC1 differently interact with miR-187 and miR-138, respectively [155]. However, extensive functional studies are generally missing for colorectal cancer.

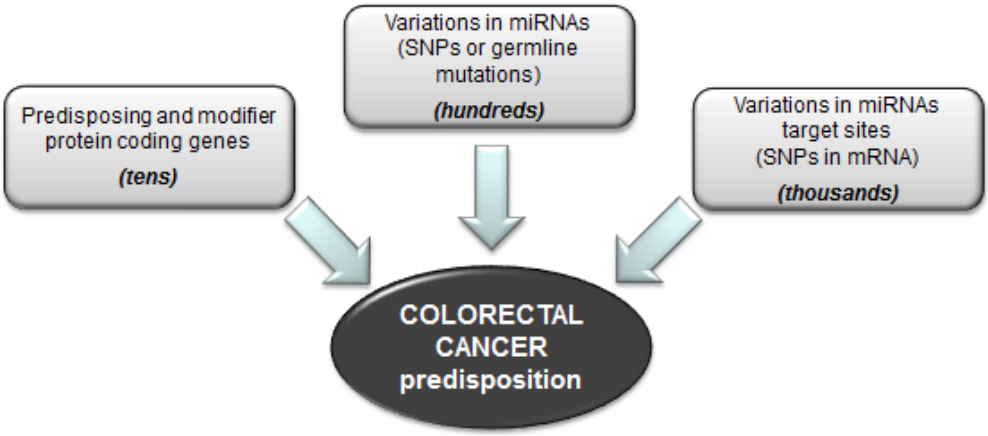


Figure 5. miRNAs and cancer predisposition (Appendix section, PAPER III, [137]).

1.2.4 Screening Methods and Diagnostic Tools

Individuals at risk should be subject to screening that might include colonoscopy, flexible sigmoidoscopy, computed tomography colonography and double-contrast barium enema for detection of polyps and cancer, and high-sensitive fecal occult blood test (FOBT), fecal DNA test and fecal immunochemical test for detection of cancer [156]. Colonoscopy is nowadays one of the gold standard methods for colorectal cancer screening [157]. However, it is an invasive technique which causes inconveniences for the patients and it is discouraged, under a public-health perspective, for the entire population [157]. Non-invasive methods are available and are commonly used, such as the FOBT, which is performed in patient stool.

miRNAs are detected and are relatively stable in stool and therefore may serve as potential diagnostic tools for the future. In 2009, Ahmed *et al.* detected seven miRNAs upregulated (miR-21, miR-106a, miR-96, miR-203, miR-20a, miR-326, and miR-92) and seven miRNAs downregulated (miR-320, miR-126, miR-484-5p, miR-143, miR-145, miR-16 and miR-125b) in stool and tissue of colorectal cancer patients compared with controls [158]. miR-21 was validated in two other studies [159, 160]. Link *et al.* study showed that miR-21, as well as miR-106a, have a higher expression in stool specimens from colorectal cancer (or adenoma) than in control group [159]. Wu *et al.* found that miR-21 and miR-92a were significantly increased in feces of colorectal

cancer patients, although only miR-92a, but not miR-21, was higher in stool from patients with polyps than controls [160]. Koga *et al.* isolated exfoliated colonocytes from feces and found increased expression of miR-17-92 cluster and miR-135 in colorectal cancer patient samples than in healthy controls [161]. In 2011, two interesting studies were published by Bernardini group [162, 163]. The first demonstrated that epigenetic silencing of miR-34b/c through hypermethylation was present in 97.5% of primary colorectal tumors [162]. Afterwards, the authors analyzed if this result could also be found in the stool of the patients and detected hypermethylation of miR-34b/c was found in 75% of fecal samples [162]. In the second study, the authors suggested miR-144* as a potential colorectal cancer marker based on investigations in stool from patients [163].

Besides fecal samples, and as previously stated, miRNAs can also be found in circulating plasma/serum and its screening can contribute to colorectal cancer diagnosis. Deregulated miRNA in plasma may indicate the presence of colorectal cancer. Chen *et al.* was the first to look into miRNAs in plasma from colorectal patients and found that 69 miRNAs were detected in colorectal cancer but not in the normal control group [164]. Ng *et al.* used Real-Time Polymerase Chain reaction (RT-PCR) to examine the expression levels of miRNAs in plasma samples collected from colorectal cancer patients and normal controls and found that miR-17-3p and miR-92 were elevated in the plasma of colorectal cancer patients [165]. Notably, plasma levels of these miRNAs were reduced 7 days after tumor surgical removal, when compared with the pre-operatory levels, proving circulating miR-17-3p and miR-92 are of tumor origin [165]. Using an independent set of samples, the authors further determined that plasma miR-92 expression levels in colorectal cancer patients were different from gastric cancer and inflammatory bowel disease and from normal subjects [165]. The impact of miRNA expression levels in plasma as a diagnostic tool could be increased if differences were detected at early stages of the disease as these have a higher probability of survival than late disease stages [166]. In Ng *et al*. study, the authors detected as increased miR-92 expression level already in TNM stage I, compared with normal controls; however, the number of cases used in this study was low [165, 166]. Huang *et al.* focused his work on evaluating miRNAs in plasma that could serve as early

detection tools for colorectal cancer [167]. Firstly, the authors found miR-29a and miR-92a significantly upregulated in plasma from colorectal patients comparing with controls [167]. The authors did not consider miR-17-3p, detected in Ng *et al.* study as a potential biomarkers, because it levels were very low to be detected by RT-PCR. Secondly, the same miRNAs were found to be upregulated in advanced adenomas compared with normal controls, proving the diagnostic value in early lesions of colorectal cancer development [167]. Pu *et al.* study focused solely on 3 miRNAs. The authors found miR-221 plasma levels to be upregulated in colorectal cancer patients (compared with normal controls). Noteworthy, miR-211 is not only a potential biomarker for colorectal cancer diagnosis but its´ plasma levels are also a predictor of the overall survival in colorectal cancer [168]. Furthermore, Chen *et al.* experiments demonstrated that high plasma levels of miR-141 are associated with stage IV colon cancer and that miR-141 plasma levels correlate with poor survival [169]. The authors suggested circulating miR-141 might be a potential novel biomarker for metastatic colon cancer [169]. Moreover, Wang *et al.* study aimed to find miRNA plasma markers for colorectal cancer metastasis. miR-29a levels in serum were significantly higher in liver metastases of colorectal cancer patients than in non-metastatic colorectal patients, providing evidences of a potential biomarker for early detection of colorectal liver metastasis [170]. In conclusion, analysis of expression levels of a panel of miRNAs present in colorectal cancer patients' feces or bloodstream may be a tool for diagnosis and prognosis of this disease.

1.2.5 Adenoma-Carcinoma Sequence and the Serrated Polyp Pathway

Traditionally, colorectal cancers are believed to arise from epithelial polyps, named neoplastic polyps or adenomas, following an adenoma – carcinoma sequence [171, 172]. Adenomas have potential malignancy and are regarded as precursors of most colorectal cancers [171]. Classically, in a model proposed by Fearon *et al.* in 1990 [173], evolution of colorectal cancer was understood as linear sequence of steps, with the earliest genetic alteration being APC inactivation [174]. APC is a tumor suppressor gene that, together with glycogen synthase kinase 3 β (GSK-3ß) and Axin proteins,

regulate free β-catenin [175]. Binding of β-catenin to this complex leads to β-catenin phosphorylation and consequently it is rapidly degraded [175]. Upon APC loss of function, unphosphorylated β-catenin accumulates in the cell and after translocation to the nucleus, it binds to transcription factors of the T-cell factor (TCF)/lymphoid-enhancer factor (LEF) family leading to an increased expression of several specific target genes including c-Myc, cyclin D1, MMP-7, and ITF-2 (canonical Wnt pathway) [175]. Mutations in APC, Axin or β-catenin all lead to deregulation of Wnt signaling [175]. Wild-type APC is also able to downregulate non-phosphorylated oncogenic forms of β-catenin. However, in colorectal cancer, APC loss of function is much more common than β-catenin mutations [175]. About 60% to 80% of sporadic adenomas and adenocarcinomas harbor somatic APC mutations (the majority of which generates premature stop codons and consequently truncated proteins; though missense mutations can also been found in a very reduced percentage) [176]. Loss of heterozygosity (LOH) in chromosome 5q, where APC gene is located, have been found [173, 174]. Approximately 20% percent of colorectal adenocarcinoma lost one of the alleles in chromosome 5q that was present in normal tissue [174, 177]. Of note, the main hallmark of FAP is a germ-line mutation of APC gene. Moreover, miRNAs can regulate APC levels. miR-135a and miR-135b, which are highly expressed in colorectal adenomas and carcinomas, regulate APC by binding to its 3´UTR and induce B-catenin signaling [178]. On the other hand, miRNAs can also be downstream targets of APC. Using a colon cell line and comparing miRNA levels before and after restoration of APC function, Wang *et al.* could find 6 upregulated miRNAs and 8 downregulated miRNAs, among which is miR-122a [179].

As previously stated, APC mutation is the earliest genetic alteration in the genesis of colorectal cancer being found in the earliest stages of the adenoma–carcinoma sequence (in the aberrant crypt foci stage) [174]. Then, mutations in KRAS gene, possibly together with events affecting other genes, contribute to adenoma growth [173, 174]. KRAS mutations are found in approximately 50% of sporadic colorectal adenomas and carcinomas [174] and confer constitutive activation of the Ras/MAPK pathway [180]. KRAS can be targeted by several miRNAs. This is the case of miR-143 that, in colorectal cancer, was experimentally proven to directly target KRAS

[181]. miR-143 mimics inhibit KRAS expression and consequently inhibits constitutive phosphorylation of ERK1/2 [181]. In contrast, miRNAs expression profiles can be differentiated according to the KRAS status. Particularly, increased expression of miR-127-3p and miR-92a is observed in KRAS mutant tumors compared with KRAS wild-type tumors [182]. Later in the progression stages, LOH in chromosome 18q, where two members of TGF-β pathway, SMAD2 and SMAD4, are located, and in chromosome 17p, where TP53 tumor suppressor gene located, and are found in late progression stages [174]. About 75% of 17q loss is found in carcinoma while less than 5% is found in early adenoma [173]. Interestingly, miR-34a, a miRNA downregulated in colon cancer, is regulated by P53; miR-34 in turn targets SIRT, which interacts with p53 and mediates deacethylation of its Lys382 residue, causing a decrease in p53-mediated transcriptional activation and reduced the downstream protein such as p21 and PUMA levels, generating therefore a miR-34-SIRT-P53 feedback loop [183]. Importantly, miR-34 links p53 to WNT pathway, since the p53-regulated miRNA-34 targets Wnt genes resulting in suppression of TCF/LEF transcriptional activity [184]. In conclusion, regarding colorectal carcinomas arising from conventional adenomas through the adenoma-carcinoma sequence, APC mutation is considered an early event and it is followed by several key players as KRAS mutation, or SMAD2/4 and P53 loss, among others. miRNAs are implicated as both regulators and downstream targets of these players [185]. Some of the miRNAs involved in colorectal cancer tumorigenesis and progression are represented in Figure 6.

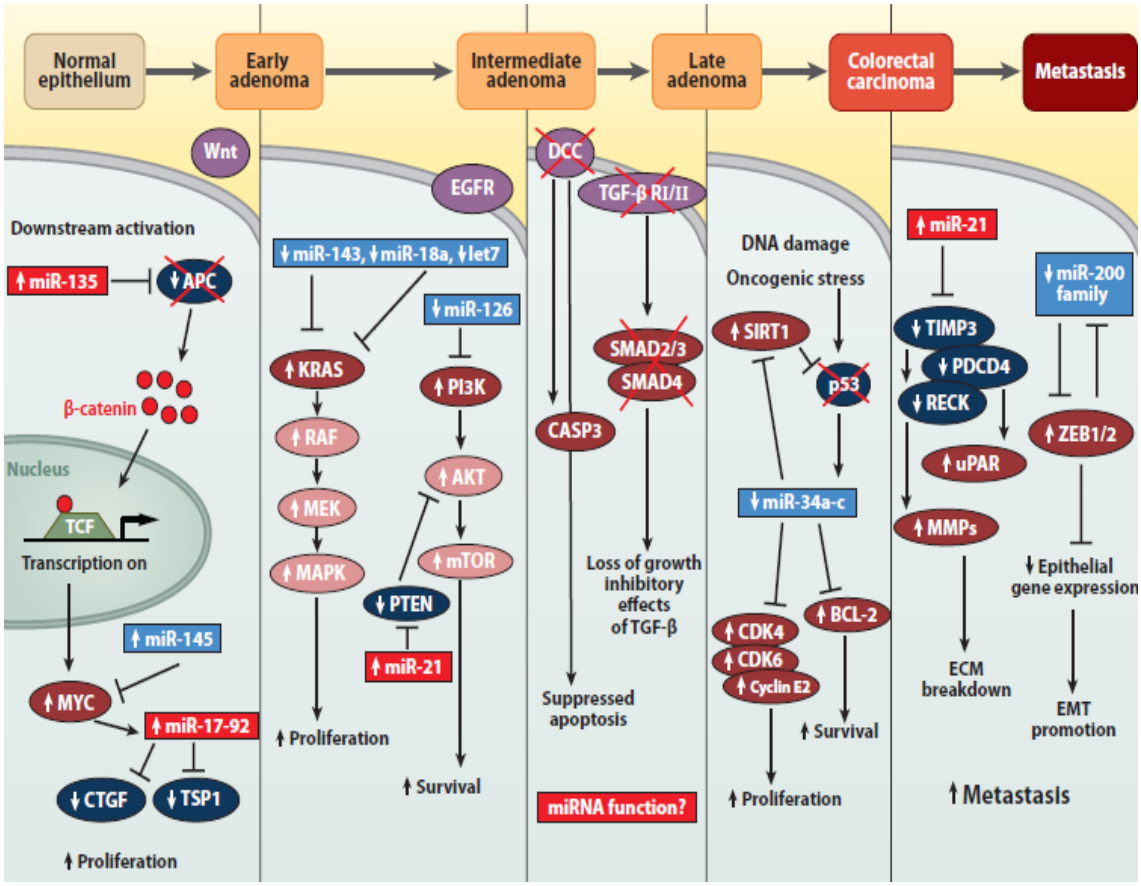


Figure 6. Example of some miRNAs as regulators of major players in colorectal cancer progression (adapted from Fearon et al., [185])

Alternatively to the adenoma-carcinoma sequence, increasing evidences pinpoint cancers that arise via the serrated polyp pathway (approximately 15-20% of all colorectal tumors) that implicate the previously thought non-premalignant hyperplastic polyps as potential precursors of colorectal cancer [171, 172]. In fact, colorectal tumors are heterogeneous and serrated carcinomas are clinically, morphologically, and molecularly distinct from colorectal cancers originating via adenoma-carcinoma. Serrated polyps comprise typical hyperplastic polyps (most common serrated polyps, comprising 80–90%), sessile serrated adenomas (atypical hyperplastic polyps variants), and dysplastic serrated polyps [171, 186]. Adenoma, are distinguishable at the microscopic level from hyperplastic polyps and the cancers caused by the latest are more likely to be found in females, located in the proximal colon and occur later than the cancers of adenoma origin [171, 187]. Some of the

carcinomas arising via the serrated pathway have mutations in BRAF, a gene that plays a role in regulating the MAP kinase/ERKs signaling pathway [188]. KRAS works in the same pathway and BRAF and KRAS mutations are usually mutually exclusive [188]. While BRAF mutations were found to be much more frequent in sessile serrated adenomas and mixed polyps when compared with traditional hyperplastic polyps, and are rarely found in conventional adenomas, KRAS mutations are more commonly associated with the adenoma-carcinoma sequence [188]. Lesions bearing BRAF mutations are prone to methylation of the CpG island promoter regions resulting in the epigenetic silencing of a number of genes [188, 189].

1.2.6 Molecular Classification and Genetic Pathways

The pathogenesis of colorectal cancer involves distinct genetic pathways: the microsatellite instability (MSI) and the chromosome instability (CIN) pathways, which are described in sub-chapter 1.2.6.1 and 1.2.6.2, respectively [190]. Additionally, a more recent unique subset of colorectal cancer was established according to the tumors´ degree of methylation, named CpG island methylator phenotype (CIMP), described in sub-chapter 1.2.6.3 [191]. All these pathways contribute to the acquisition of genomic instability.

1.2.6.1 Microsatellite Stable and Microsatellite Unstable Tumors and DNA Mismatch Repair System

DNA mismatch repair (MMR) system is responsible for correction of errors that occur during DNA cell replication [192]. This system involves mainly 4 processes including, surveillance of base-base mismatches or insertion-deletion loops, removal of these lesions, correction of the sequence, and religation to DNA [193]. Components of the MMR system are conserved from prokaryotic or single-cell eukaryotic organisms to humans. MMR system functions as hetereodimer complexes. The complex hMutS recognizes mismatches and is characterized by 2 major forms: hMutSα, constituted by the dimer MSH2-MSH6 that has a higher affinity to recognize base-base mismatches

and short insertion-deletion loops; and hMutSβ, constituted by MSH2-MSH3 that mainly detects large mismatches. When hMutS binds DNA lesions, it recruits the complex hMutL. This last complex may be presented in 3 different forms according to the protein that dimerizes with MLH1: hMutLα (heterodimer MLH1-PMS2), hMutLβ (heterodimer MHL1-PMS1), hMutLγ (MLH1-MLH3) [193, 194, 195]. Failure of the MMR function can be caused by either mutations or deletions or epigenetic silencing [196]. As MSH2 and MLH1 are the common components of hMutS and hMutL, respectively, loss of either proteins abolishes MMR activity [196]. Mutation rates in cancer cells with a deficient MMR system are 100–1,000 fold greater than in normal cells [193].

Microsatellites are tandem repeats of short DNA sequences (1-6 nt) distributed throughout the genome. Microsatellite instability (MSI) is caused by loss of DNA repair activity [194]. Therefore, when DNA MMR system is not properly functioning, insertions and deletions in the microsatellite regions (that are especially susceptible to accumulation of mutations since DNA polymerases cannot bind DNA efficiently) are not repair, causing an expansion or contraction of the microsatellites [192, 195]. This is known as MSI (or MIN) [195]. This phenomenon has been observed not only in colorectal tumors but also in other tumor types, including gastric, endometrial, ovarian, sebaceous carcinomas, glioblastoma, lypmphomas and medulloblastoma [192, 197]. As MSI is the indicator of defects in the DNA MMR system, a panel of microsatellite markers is indicated for tumor MSI diagnosis, according to the Revised Bethesda Guidelines [198, 199]. According to this, tumors can be classified as MSI-high (MSI-H), MSI-low (MSI-L) and microsatellite stable (MSS) [200]. Tumors with MSI-H are distinctly characterized from MSI-L and MSS, in particular, MSI-H tumors tend to be poorly differentiated, mucinous, histologically heterogeneous formed in the proximal colon, have an expansile growth pattern, and an increase tumor infiltrating lymphocytes with a prominent inflammatory reaction [200]. Two algorithms based on clinical and histologic parameters have been developed to predict MSI-H colorectal cancer tumors, namely ‘‘MsPath’ and ‘‘MSI probability score” models [195, 201, 202]. Importantly, MSI tumors have a slightly better prognosis than other types of colorectal cancer and respond differently to chemotherapeutics [194, 200]. MSI-H frequency on

colorectal sporadic tumors is 10-15% [200]. In sporadic cases, instability is usually caused by hypermethylation of MLH1 promoter. The loss of MLH1 expression increases with age [194]. Moreover, 3% of all colorectal cancers are MSI tumors associated with Lynch syndrome [194]. This is caused by germline mutations in MLH1, MSH2, MSH6 and PMS2 [192]. In addition, although it was thought to be exclusive from MSI-sporadic tumors, a small subset of tumors derived from Lynch syndrome also have MLH1 epigenetic inactivation by hypermethylation of its promoter [196]. Considering the Knudson two-hit hypothesis of tumorigenesis, in Lynch syndrome the "first hit" is, as mentioned above, a germline mutation and the "second hit" may occur through deletion, gene conversion, methylation or point mutation [203]. It is also important to take into consideration that miRNAs can control mismatch repair levels. Valerie *et al.* suggested that this can be one of the mechanisms to explain the 5% MSI cases not justified by MMR mutations/epigenetic inactivation [204]. The authors showed that miR-155, which is overexpressed in colorectal cancer specimens compared with adjacent normal tissue, targets hMLH1, hMSH2, and hMSH6 and that it overexpression induces a mutator phenotype and MSI [204]. Further support of this hypothesis is the fact that miR-155 expression is inversely related to MLH1 and MSH2 proteins expression in human colorectal cancer tissues [204].

Nowadays, it is common to apply MSI tests and perform MMR protein evaluation by immunohistochemistry in order to screen for MSI-tumors and Lynch syndrome [195]. BRAF is an oncogene involved in the Ras/Raf/Map kinase pathway. A hotspot mutation in BRAF (V600E), which aminoacid change results in constitutive activation of the BRAF kinase and promotes cell transformation [174], is present in a portion of sporadic MSI-H colorectal tumors (40-50%) but it is extremely rare in colorectal cancer originated from Lynch syndrome [196]. Also, it is highly associated with MLH1 promoter methylation [203]. When MLH1 protein is lost, both BRAF (V600E) mutation test and MLH1 promoter methylation testing (though a small percentage of Lynch syndrome may present MLH1 methylation) are useful tools to discriminate between Lynch syndrome and MSI-H sporadic colorectal cancer cases [203]. Other features may help distinguishing Lynch syndrome from MSI-H sporadic colorectal cancer, as patients´ age - tumor diagnosis at less than 50 years is more

common in patients with Lynch syndrome and diagnosis at more than 70 years is more common in MSI-H sporadic colorectal cancer; patients´ gender – MSI-H sporadic colorectal cancer is more common in females than males; tumor budding – it is more frequent in Lynch syndrome tumors; serrated precursors – the frequent mucious histology and serration is probably due to their origin from serrated precursors and is an attribute of MSI-H sporadic CRC but not Lynch syndrome tumors [195].

In MSI tumors, genes that contain repetitive elements can be mutated. As a consequence, in MSI-H sporadic tumors, several tumor suppressor genes that are implicated in tumor progression, including TGFβ-RII, BAX, IGF2R, CASP5, MSH3 and MSH6, can be mutated [195, 200, 205].

Due to the different molecular characteristics between MSI and MSS tumors, it is therefore not surprising that unique miRNA signatures exist between colorectal tumors based the microsatellite instability status. The first study that addressed this issue was reported in 2007 [206]. Using microarrays, Lanza *et al.* identified 14 miRNAs distinctly expressed between MSS and MSI tumor´ groups. To point out that among these are members of the miR-17-92 family, including miR-17-5p, miR-20, miR-25, miR-92-1, miR-92-2, miR-93-1 and miR-106a, up-regulated in MSS versus MSI-H colon cancer [206]. Additionally, the study also found 451 differently expressed mRNAs and supports that expression profiles that combine miRNA/mRNA expression are a good approach to discrimination between MSI-H and MSS human colon cancer [206]. Earle *et al.* used a panel of selected miRNAs and performed RT-PCR to analyze the differences between MSI and MSS groups [207]. miR-155, -31, -223 and -26b increased relative expression was significantly associated to MSI-H (miR-31 and miR-223 in patients with Lynch syndrome) and miR-92, let-7a and miR-145 increased relative expression was associated with MSI-L, while miR-196a increased relative expression was associated with MSS status [207]. Finally, in a genome-wide miRNA expression profile analysis, Balaguer *et al.* found a subset of miRNAs whose expression was discriminated between sporadic MSI and MSS tumors, including miR-938, miR-615-5p, miR-1184, miR-551a, miR-622 (upregulated in MSI versus MSS) and miR-17-5p, miR-192*, and miR-337-3p (downregulated in MSI versus MSS) [208]. Most importantly, this study distinguished miRNA profiles in sporadic MSI and Lynch

syndrome, specifically miR-30a*, miR-16-2*, 362-5p and miR-1238 (upregulated in Lynch syndrome tumors vs sporadic MSI) and miR-622 (downregulated in Lynch syndrome tumors vs sporadic MSI) [208]. In conclusion, considering these studies, the discrepancies on the results are obvious. These inconsistences can be explained by biological differences other than MSI [208]. However, they can also originate from the different biological samples used (e.g., tumors collection time and sources, formalin-fixed paraffin-embedded and frozen samples) or the different technical approached used in the different studies (e.g., types of arrays, analysis methods) [208]. Hence, if future studies are able to overcome these pitfalls, the MSI/MSS miRNA-associated expression patterns could be translated into a useful tool for the clinics and aid in patient´s personalized treatment based on their molecular classification.

1.2.6.2 Chromosomal Instability

Another genetic signature can be found in colorectal cancer that contributes to the activation of oncogenes and inactivation of tumor suppressor genes: CIN [190]. It is the most common form of genomic instability in colorectal cancer, accounting for 80%-85% of all colorectal cancers. CIN and MSI mechanisms are generally mutually exclusive [209]. Thus, the CIN-positive tumors are MSS tumors [209]. FAP is associated to the CIN mechanism [209]. It is characterized by genetic deletions, duplications and chromosomal rearrangements and it is translated into aneuploidy. Therefore, CIN can be recognized by determining tumor DNA ploidy, through cytometry or cytogenetic techniques [190, 210]. Lee *et al.* showed that CIN-positive colorectal cell lines are more likely to exhibit multi-drug resistance than CIN-negative (diploid) colorectal cell lines [210]. The same authors showed that CIN-positive tumors confer a worse prognosis (lower overall survival and progression-free survival) than diploid tumors after patients´ treatment with cytotoxic therapy. Hence, CIN seems to confer survival and adaptation qualities to tumor cells [211]. CIN appears to arise due to defective surveillance of mitosis but the molecular basis of CIN remains largely unclear [210]. Particularly, Cahill *et al.* showed that the basis for CIN can be loss of function of mitotic checkpoints (eg, gene BUB1) [212]. Amplification of the cell cycle-regulated kinase

Aurora kinase A (29% in human colorectal tissues) may also cause CIN, by disruption of CDC4-cyclin E pathway [213]. Furthermore, APC gene mutations are likely to elicit CIN, through spindle stress [174, 211]. Other candidate for CIN origin includes APC and P53 mutations [174].

1.2.6.3 CpG Island Methylator Phenotype

Issa´s group introduced the term CIMP [214] to designate a genetic signature present in colorectal cancers, which is characterized by the exhibition of frequent and concurrent hypermethylation at specific promoter genes [215]. It is the most common source of epigenetic inactivation of the MMR gene MLH1, the main cause of the MSI-sporadic tumors [190, 216]. However, CIMP was also associated with non-MSI/non-LOH tumors, suggesting an independent pathway for tumor development [190]. Clinically, CIMP is associated with elderly female patients, proximal tumor localization, and frequent mucinous and poor differentiation [193, 216]. It is usually linked to serrated polyps rather than classical adenomas, being methylation, instead of mutation of tumor suppressor genes, the driving force [215]. Therefore, in CIMP-positive/MSI-H tumors, mutations of APC, KRAS, P53 and CTNNB are infrequent [166]. Molecularly, a consensus on the methylation markers to define CIMP-positive has not been established [215]. CIMP-positive colorectal cancers have frequent MSI. CIMP is highly associated with activating mutations in BRAF gene (V600E) [190, 215]. Although BRAF and KRAS mutations are mutually exclusive, several reports found an association between CIMP phenotype and activating KRAS mutations [190, 215]. Boland *et al.* suggests that aberrant DNA methylation is a common event of sporadic and Lynch syndrome colorectal cancers [190]. Although at the moment there are no reported studies describing miRNA expression profiles between CIMP-positive and CIMP-negative colorectal tumors, Slattery *et al.* analyzed miRNA profiles in CIMP-positive colorectal tumors and normal tissue, and found that 230 miRNA probes (comprising 129 miRNAs) had greater than 50% differential expression in CIMP-positive colon cancer versus normal tissue, while 205 miRNA probes (comprising 129 miRNAs) had

greater than 50% differential expression in CIMP-positive rectal cancer versus normal tissue [217].

1.2.7 Therapeutic Strategies (5-fluorouracil, irinotecan, oxaliplatin, cetuximab, panitumumab)

5-fluorouracil (5-FU), irinotecan and oxaliplatin are commonly used drugs for colorectal cancer. 5-FU, one of the most extensively used, is almost always administrated with folinic acid (leukovorin) to be more effective [218]. It is common that 5-FU is combined with other chemotherapy drugs. Capecitabine is an oral 5-FU prodrug [219]. Irinotecan is a topoisomerase I inhibitor that interrupts DNA replication during transcription [219]. Oxaliplatin is a second-generation platin-based drug whose mechanism of action is diverse and includes induction of DNA lesions and arrest and inhibition of nucleic acid synthesis [220]. miRNAs are involved in resistance to therapies that use these clinical agents. There are several reports showing miRNA expression differences between sensitive and resistant colorectal cancer cells to the therapeutics agents. For instances, Ross *et al.* described 5-FU drug upregulated expression levels of 19 miRNAs while downregulated the expression of 3 miRNAs [221]. Studying the effect of 5-FU and oxaliplatin on miRNA expression, there were 56 up- and 50 downregulated miRNAs significantly associated to the treatment [222]. Zhao *et al.* suggested a pathway in which 5-FU induces a decrease in c-Myc expression, which regulates miR-17-92 cluster that, by its turn, targets thrombospondin mRNA that translates into an adhesive glycoprotein that mediates cell-to-cell and cell-to-matrix interactions and plays a role in angiogenesis and tumorigenesis [223]. Core MMR components are critical players in therapy resistance. Consequently, miRNAs that regulate protein levels of these genes may contribute to resistance. This is the case of miR-21 that induces resistance to 5-FU by targeting hMSH2 [224]. Additionally, miRNAs involved in cell cycle may be implicated in drug resistance. In response to 5-FU, miR-19b was overexpressed in 5-FU-resistant colorectal cells (DLD-1 derived cell line) and its targets are predicted to be involved in cell cycle [178]. Akao *et al.* used the same cell line to show downregulation of miR-34a in 5-FU-resistant DLD-1 cells versus the

parental cells [225]. miR-20a can modulate the resistance to 5-FU, oxaliplatin and teniposide by targeting BNIP2 [226], and miR-19b can be a potential indicator of chemosensitivity to the common 5-FU-based chemotherapy regimen by targeting BIM, a key mediator of chemotherapy-induced cell death [227]. miR-192/miR-215 targets cell cycle progression in colorectal cancer by decreasing S-phase cells and these miRNAs are suggested to be important in 5-FU resistance [228]. Experiments performed in HCT116 colorectal cell line revealed that miR-143, besides reducing cell viability and increasing apoptosis, also increases sensitivity of these cells to 5-FU, through the regulation of proteins involved in ERK5/NF-kB pathways [229]. The same cell line was used to prove that anti-miR-31 by itself has no effect on cell proliferation but when combined with 5-FU it is able to inhibit proliferation [230]. One source of treatment-resistant cells are cancer stem cells (CSC) and Bitarte *et al.* explored whether miRNAs´ modulation could influent CSC drug-resistance [231]. In fact, colonospheres with properties of CSCs derived from colon carcinoma cells had miR-451 downregulated levels that, when restored, caused chemoresistance to irinotecan [231].

Epithelial growth factor receptor (EGFR) is a transmembrane glycoprotein receptor tyrosine kinase of about 170,000 kDa, also known as HER1 [2312 233]. Upon ligand binding, EGFR stimulates a downstream cell signaling cascade that regulates proliferation, apoptosis and metastasis [231]. Activation of EGFR triggers two main signaling pathways, RAS/RAF/MEK/ERK and PI3K/AKT, and also Src tyrosine kinases, PLCγ, PKC and STAT. EGFR is overexpressed in several epithelial tumors and is critical for tumor development [233]. Accordingly, expression levels of two miRNAs controlled by EGFR, namely miR-143 and miR-145, are downregulated in colorectal tumors [234]. Zho *et al.* showed that blockage of EGFR caused upregulation of miR-143 and miR-145 in a colorectal cancer cell line [234]. Additionally, transfection of miR-143/miR-145 inhibits epithelial growth factor (EGF, a EGFR ligand)-induced proliferation and DNA synthesis [234]. Cetuximab and panitumumab are two anti-EGFR monoclonal antibodies, which target the extracellular domain of the receptor and inhibit ligand-dependent EGFR signal transduction [233, 235]. Both antibodies are approved by the US Food and Drug Administration (FDA) and are effective in metastatic colorectal

cancer, but response rates are limited [232, 236]. In addition, tyrosine kinases inhibitors, such as gefitinib (FDA approved only for a small cohort of non-small-cell lung carcinoma [NSCLC] patients) and erlotinib (FDA approved as monotherapy for the treatment of NSCLC patients who did not respond to at least one prior chemotherapy, or in combination with gemcitabine for advanced pancreatic cancer patients who have not received previous chemotherapy), are able to specifically inhibit EGFR, by blocking the ligand-induced receptor autophosphorylation, but are presently not FDA approved for colorectal cancer treatment [233]. As EGFR mutations are rare in colorectal cancer, the main mechanism of resistance to cetuximab and panitumumab is mutations in KRAS, an oncogene that plays a critical role as downstream effector of EGFR signaling. KRAS mutations occur in approximately 30% to 40% of colorectal cancer patients [236]. Currently, KRAS mutation status (most commonly in codon 12 and 13) defines which patients will benefit from the anti-EGFR antibodies therapy, since the treatment benefits those patients those tumors lack KRAS activating mutations [232, 236].

Furthermore, the mutation V600E in the BRAF gene, which is one of the main effectors of KRAS, has been shown to impair responsiveness to cetuximab and panitumumab in patients with metastatic colorectal cancer [174, 237]. Nicolantonio *et al.* studied metastatic colorectal cancer patients treated with cetuximab or panitumumab and showed that none of the BRAF-mutated patients responded to treatment and had a significantly shorter progression-free survival than the other patients, while none of the responders carried BRAF mutations [238]. Additionally, in the PI3K pathway, mutations in PIK3CA (present in 20% of colorectal cancers) can constitutively activate its kinase activity and KRAS signaling pathways and consequently interfere with the response to cetuximab and panitumumab [174, 236]. In the same pathway, the main negative regulator, the tumor suppressor PTEN, is lost in 19% to 24% of colorectal cancers [236]. Loss of PTEN also correlates with patients´ reduced response to cetuximab-based therapy. In the largest cohort analyzed to date of patients with chemotherapy-refractory metastatic colorectal cancer treated with cetuximab plus chemotherapy, De Roock *et al.* found that together with KRAS, BRAF and PIK3CA mutations, also NRAS mutations were significantly associated with a low response rate to cetuximab [239]. Furthermore, an antibody can be used as a strategy

to oppose angiogenesis [240]. This antibody is named bevacizumab and is directed against VEGF [240]. Unlike cetuximab, in which the KRAS gene status can be used to determine who will benefit from the treatment, bevacizumab lacks a known predictive marker of efficacy [240].

Ragusa *et al.* tested whether miRNAs could be involved in resistance to cetuximab by analyzing miRNA differences in two colon cell lines, one sensitive and other resistant to the drug. Within the differently expressed miRNAs, the authors found 12 miRNAs predicted to target mRNAs reported to be involved in either the cetuximab pathway or the colorectal cancer [241]. Some of these miRNAs could be considered as candidate molecular markers for cetuximab resistance [241]. The authors also found miR-146b-3p and miR-486-5p to be more abundant in KRAS–mutated samples with respect to wild-type ones [241].

It is well known that miRNAs are involved in drug resistance [242]. We can hypothesize that therapeutic modulation of miRNA levels could be a way to overcome drug resistance. For instance, if in cancer a miRNA that regulates a protein involved in blocking drug activity is suppressed, it is expectable that restoring miRNA levels would decrease the expression of that specific drug-resistance protein. Also, the opposite mechanism can be postulated – if in cancer a miRNA that target genes involved in the induction of cell death by the drug is upregulated, it is necessary to suppress it so that we can increase the malignant cell sensitivity to the drug and consequently decrease cell survival and resistance to a particular chemotherapeutic regimen. Importantly, not only the levels of miRNAs but also the levels of ceRNAs should be considered. In conclusion, the concept of miRNA pharmacogenomics is a novel and promising field of research that holds new possibilities for medical therapy, including for colorectal cancer [242].

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2. Strand-specific miR-28-5p and miR-28-3p have distinct effects in colorectal cancer cells

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Strand-Specific miR-28-5p and miR-28-3p Have Distinct Effects in Colorectal Cancer Cells

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BACKGROUND & AIMS: MicroRNAs (miRNAs) can promote or inhibit tumor growth and are therefore being developed as targets for cancer therapies. They are diverse not only in the messenger RNAs (mRNA) they target, but in their production; the same hairpin RNA structure can generate mature products from each strand, termed *5p* and *3p*, that can bind different mRNAs. We analyzed the expression, functions, and mechanisms of miR-28-5p and miR-28-3p in colorectal cancer (CRC) cells. **METHODS:** We measured levels of miR-28-5p and miR-28-3p expression in 108 CRC and 49 normal colorectal samples (47 paired) by reverse transcription, quantitative real-time polymerase chain reaction. The roles of miR-28 in CRC development were studied using cultured HCT116, RKO, and SW480 cells and tumor xenograft analyses in immunodeficient mice; their mRNA targets were also investigated. **RESULTS:** miR-28-5p and miR-28-3p were down-regulated in CRC samples compared with normal colon samples. Overexpression of miRNAs in CRC cells had different effects and the miRNAs interacted with different mRNAs: miR-28-5p altered expression of *CCND1* and *HOXB3*, whereas miR-28-3p bound *NM23-H1*. Overexpression of miR-28-5p reduced CRC cell proliferation, migration, and invasion in vitro, whereas miR-28-3p increased CRC cell migration and invasion in vitro. CRC cells overexpressing miR-28 developed tumors more slowly in mice compared with control cells, but miR-28 promoted tumor metastasis in mice. **CONCLUSION: miR-28-5p and miR-28-3p are transcribed from the same RNA hairpin and are down-regulated in CRC cells. Overexpression of each has different effects on CRC cell proliferation and migration. Such information has a direct application for the design of miRNA gene therapy trials.**

Keywords: Transcript Regulation; Gene; RNA Processing.

Colorectal (CRC) cancer is the third most commonly diagnosed cancer in men and the second in women.[1] In the United States, it is the third leading cause of death by cancer, with 51,371 estimated deaths and 142,570 estimated newly diagnosed cases in 2010.[2] Therefore, new therapeutic approaches and prognostic markers are needed. In 2002, new players in cancer biology were identified: microRNAs (miRNAs).[3] These are a large family of small noncoding RNAs with approximately 20-nt length that regulate gene expression post-transcriptionally by inhibition of translation or messenger RNA (mRNA) degradation.[4] miRNA targeting occurs by binding to 3′-untranslated regions, coding sequences, or 5′-untranslated regions of target mRNA that can be involved in diverse biological processes, such as proliferation, apoptosis, inflammation, differentiation, and metastasis.[4] miRNAs can function as either oncogenes or tumor suppressor genes, depending on the type of tumor or the cellular context.[5] In CRC, miRNAs have been involved in tumor susceptibility (as polymorphisms in miRNA-binding sites have been associated with CRC risk) and in diagnosis (as miRNAs can be detected in feces or blood and used as biomarkers).[6] In addition, miRNA expression is dysregulated in CRC, as well as in other cancer types, and miRNAs have emerged as potential new therapeutic targets.[6,7] Therefore, understanding the role of miRNAs in CRC is crucial for the development of new therapies.

In the miRNA biogenesis pathway, long primary transcripts transcribed from the genome are processed by the cellular RNase enzyme III Drosha into a structure of 60 to 110 nt called precursor miRNA (pre-miRNA), which is then exported to the cytoplasm by an Exportin 5-dependent mechanism.[4] The pre-miRNA is cleaved by the RNase III enzyme Dicer-1 producing a short, imperfect, double-stranded miRNA duplex, which is unwound by a helicase, creating a mature miRNA.[4] In some cases, 2 mature miRNAs can be excised from the same stem-loop pre-miRNA.[8] These 5p and 3p miRNAs, although generated from a single primary transcript, have different sequences and therefore tar-

Abbreviations used in this paper: CRC, colorectal cancer; miRNA, microRNA; MSS, microsatellite stable; mRNA, messenger RNA; MSI, microsatellite unstable; PCR, polymerase chain reaction; SCR, scrambled negative control; PARP1, poly(adenosine diphosphate-ribose) polymerase 1; pre-miRNA, precursor miRNA.

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get different mRNAs. In humans, 2 different mature miRNA sequences are excised from opposite arms of the stem-loop pre-miR-28 and generate 2 different miRNAs—hsa-miR-28-5p and hsa-miR-28-3p. Despite nearly a decade of studies on miRNA roles in cancer,[3] the comparative roles of strand-specific mature miRNAs that originated from the same stem-loop precursor (5p and 3p) have not yet been fully studied.

To our knowledge, the roles miR-28-5p and miR-28-3p play in CRC have never been described. Therefore, the purpose of our study was to analyze miR-28-5p and miR-28-3p expression and to use in vitro and in vivo approaches to understand, for the first time, the functions and mechanisms of these 2 miRNAs in CRC.

Materials and Methods

Colorectal Samples

Eighty-five CRC samples and 26 normal colorectal tissue samples (of which 24 were paired) were collected between 2003 and 2008 at the University Hospital of Ferrara in Ferrara, Italy (first sample set). Forty-two tumors were classified as microsatellite stable (MSS), and 43 tumors were classified as microsatellite unstable (MSI) (Supplementary Methods). For a confirmation set of samples, we obtained 23 paired samples of tumor and adjacent colorectal tissue that were collected between 2002 and 2005 at the Istituto per lo Studio e la Cura dei Tumori della Romagna in Meldola, Italy (second sample set). Tumors were classified according to the World Health Organization pathologic classification system. All patients provided informed consent, and collection of the samples was approved by the institutional review board at each institution. Patients did not receive any therapy before surgery. Upon resection, fresh surgical specimens were immediately snap-frozen in liquid nitrogen and stored at −80°C. Total RNA from tissue samples was isolated using Trizol reagent (Invitrogen, Carlsbad, CA), according to manufacturer's instructions (Supplementary Methods).

Reverse Transcription Quantitative Real-Time Polymerase Chain Reaction

RNA purity was assessed by measuring absorbance at 260, 280, and 230 nm. Mean 260/280 ratio was 1.97 ± 0.05, with a range between 1.86 and 2.05, and mean 260/230 ratio was 2.17 ± 0.11, with a range between 2.00 and 2.31. In addition, as recommended by the Minimum Information for Publication of Quantitative Real-Time PCR Experiments guidelines,[9,10] we analyzed RNA integrity by gel electrophoresis and clearly defined 28S and 18S ribosomal RNA bands were visualized. Samples with low quality that did not meet these criteria were excluded. We quantified miR-28-5p and miR-28-3p expression with real-time quantitative polymerase chain reaction TaqMan miRNA assays (Applied Biosystems, Foster City, CA), namely assay 000411 for miR-28-5p, assay 002446 for miR-28-3p, and assay 001973 for U6 snRNA (Supplementary Methods). The efficiency of the Taqman assays used in this study was determined (Supplementary Figure 1 and Supplementary Table 1). Relative expression levels were calculated using the $\Delta\Delta C_t$[11] and the Pfaffl method.[12]

In Vitro Cell Proliferation Assays

HCT116 and RKO cells transfected with scrambled negative control (SCR), miR-28-5p, or miR-28-3p were seeded onto a 12-well plate at 1×10^5 cells/well in triplicate. Cells were harvested and counted at 0, 24, 48, 72, and 96 hours after transfection using the Vi-CELL cell viability analyzer (Beckman Coulter, Brea, CA). In order to further confirm our results, a 3-(4,5-dmethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay was performed (Supplementary Methods). The experiment was repeated twice independently.

In Vitro Cell Migration and Invasion Assays

To determine the effect of miR-28-5p and miR-28-3p on cell migration, we used 6.5-mm diameter Transwell chambers with 8-μm pore size polycarbonate membranes (Corning Incorporated, Lowell, MA). To determine the effect of these miRNAs on cell invasion, we used BioCoat growth-factor reduced Matrigel invasion chambers (BD Biosciences, Bedford, MA). Cells transfected with SCR, miR-28-5p, or miR-28-3p were resuspended in serum-free medium and plated on the top of the Transwell chambers. Fetal bovine serum was used as a chemoattractant on both assays. Each assay was performed in triplicate and in 2 independent experiments. Additional details are described in Supplementary Methods.

In Vivo Studies of Tumorigenesis and Metastatic Potential

For the in vivo tumorigenesis assay, 1.5×10^6 HCT116-pBABE-miR-28 or HCT116-pBABE-empty cells were subcutaneously injected into the flanks of NOD-SCID-IL2R−deficient mice (n = 9; stock #005557; The Jackson Laboratory, Bar Harbor, ME). Tumor size was measured every 2 days. Animals were sacrificed 21 days after injection, and final tumor volume was determined. Tumor size was determined by digital caliper measurements (length and width in mm), and tumor volume (mm^3) was estimated using the following formula: tumor volume = ½ (length × $width^2$).

For the in vivo tumor-metastasis assay, 4×10^6 HCT116-pBABE-miR-28 and HCT116-pBABE-empty cells were injected into the tail vein of NOD-SCID-IL2R–deficient mice (n = 11/group). Thirty-five days after injection the mice were sacrificed. All of the organs were examined at necropsy. Tumors were sectioned, stained with H&E and anti−green fluorescent protein antibody (Ab13970; Abcam, Cambridge, MA), and examined histologically.

All animal care and handling was approved by The University of Texas MD Anderson Institutional Animal Care and Use Committee.

Statistical Analysis

Shapiro–Wilk test was used to verify the clinical samples' distribution. Differences were analyzed using the nonparametric test Mann–Whitney–Wilcoxon. To compare the paired groups, paired *t* test was used. For in vitro and in vivo studies, the differences between groups were analyzed using Student *t* test (2-tailed), assuming unequal variance. Discrete variables were compared with the Fisher exact test. Graphics represent the mean ± standard deviation, unless otherwise stated. Statistical analysis was performed in R (version 2.11.0). Statistical significance was considered if $P < .05$.

Additional methods, including cell culture, STR DNA fingerprinting, and miRNA mimics transfection, apoptosis quantification, caspase activity, cell cycle analysis by flow cytometry, establishment of miR-28–expressing cell line, miRNA target prediction, Western blot, and luciferase reporter assays, are available in Supplementary Methods.

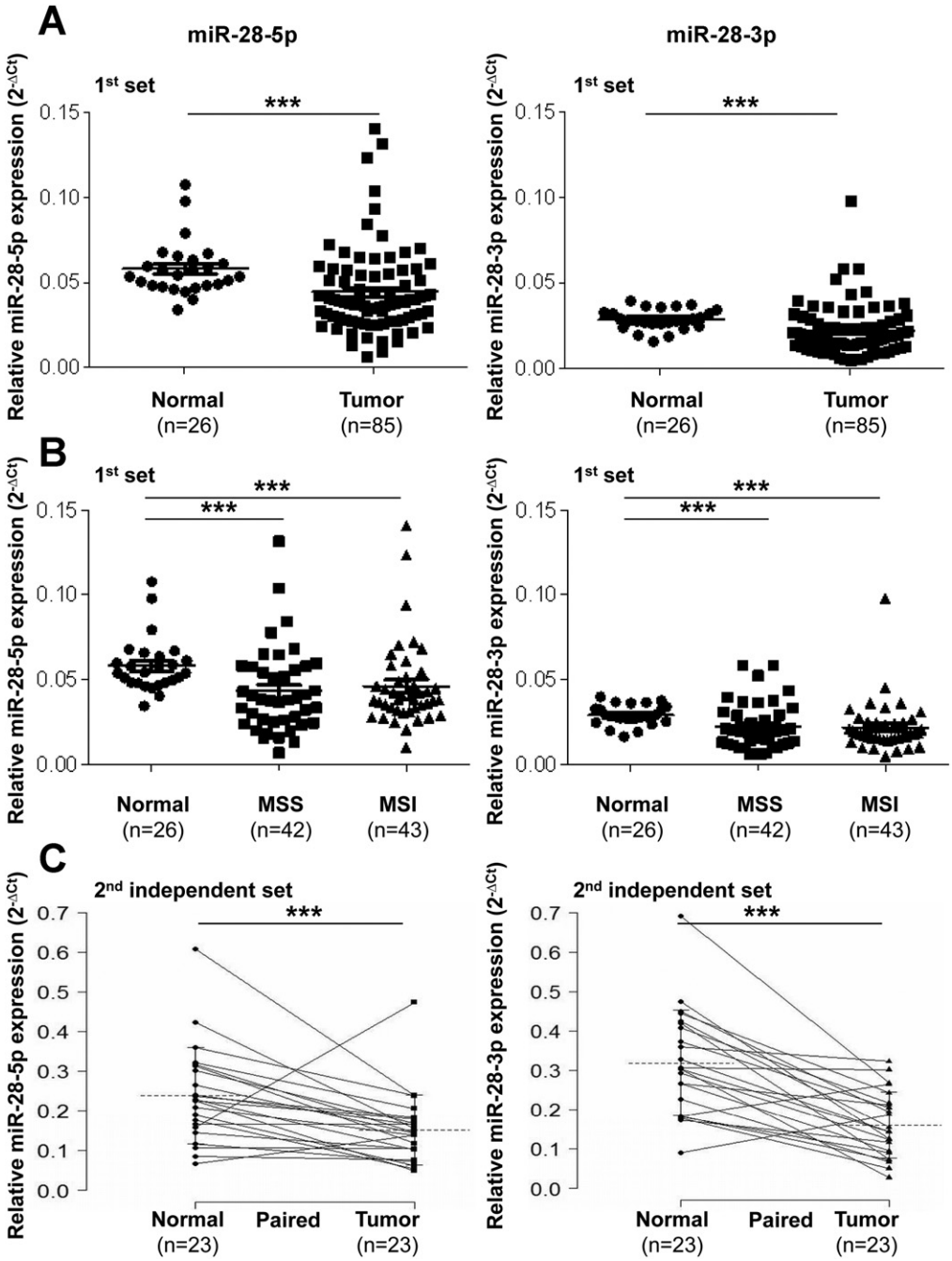

Figure 1. Expression of miR-28-5p and miR-28-3p in colon tissue samples. (*A*) Quantitative real-time PCR analysis shows that miR-28-5p and miR-28-3p are down-regulated in colon cancer samples compared with normal colorectal tissue samples. (*B*) Both MSS and MSI tumors express significantly less miR-28-5p and miR-28-3p levels when compared with normal colon tissue. No differences were found when comparing miR-28-5p and miR-28-3p levels of MSS and MSI tumors. (*C*) miRNAs down-regulation in CRC tumors paired with normal tissue from the second set of patients. All values of miRNA expression levels were normalized to small nuclear RNA U6. Mean ± standard error of the mean are represented in the images (***$P <$ .005, Mann–Whitney–Wilcoxon test, and paired *t* test for paired normal vs tumor groups).

Results

miR-28-5p and miR-28-3p Are Down-regulated in CRC

Expression levels of miR-28-5p and miR-28-3p were analyzed by quantitative real-time polymerase chain reaction (PCR) in 85 human CRC specimens and 26 normal human colorectal specimens. In order to ensure that the reference gene snRNA U6 does not change between normal and tumor samples, we calculated the mean C_t values as 2^{-Ct}. Levels of U6 did not differ between normal and tumor tissue, $2^{-CtTumor}/2^{-CtNormal} = 0.94$ ($P =$ .41) (Supplementary Figure 2). Both miRNA-28-5p and miR-28-3p were significantly down-regulated in CRC samples (miR-28-5p, $P <$ .005; miR-28-3p, $P <$ .005) (Figure 1*A*). Both MSS (n = 42) and MSI (n = 43) tumors showed down-regulation of miR-28 expression compared with the normal colon tissue (miR-28-5p normal vs MSS, $P <$ .005 and normal vs MSI, $P <$ .005; miR-28-3p normal vs MSS, $P <$ .005 and normal vs MSI, $P <$ .005); however, no significant differences between MSS and MSI tumors were found (miR-28-5p MSS vs MSI, $P =$ .418; miR-28-3p MSS vs MSI, $P =$ .996) (Figure 1*B*). We also analyzed the expression of these miRNAs in the subset of 24 pairs of normal and tumor tissue samples from the same patients, and in agreement with these data, we found significant down-regulation of miR-28-5p and miR-28-3p in CRC

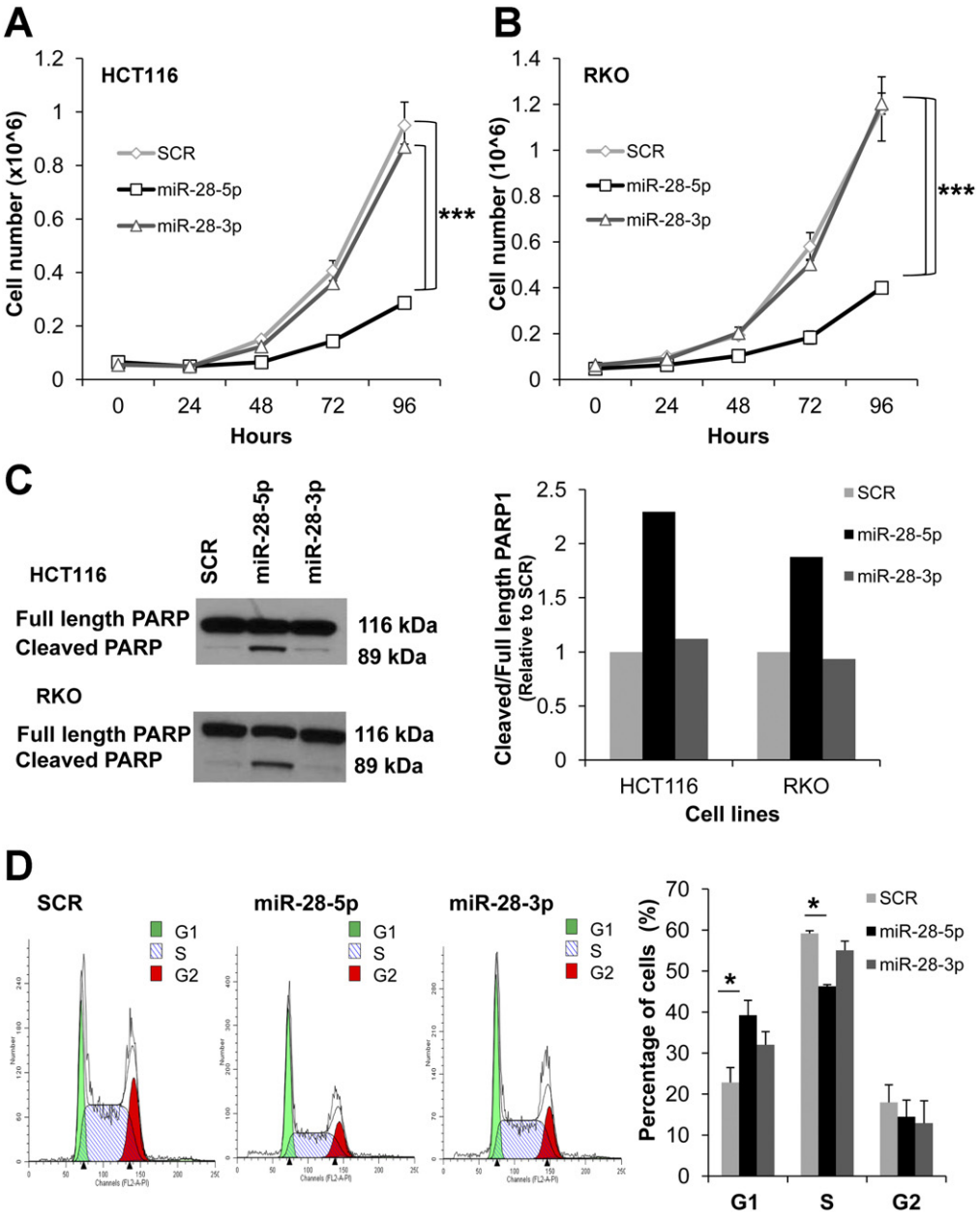

Figure 2. Biological effects of miR-28-5p in proliferation, apoptosis, and cell cycle in vitro. (*A*, *B*) Representative experiment of the proliferation effect of miR-28-5p and miR-28-3p in HCT116 and RKO colon cell lines. Cell numbers were counted every 24 hours for 4 days post-transfection with SCR, miR-28-5p, or miR-28-3p. miR-28-5p, but not miR-28-3p, inhibited growth in both HCT116 and RKO cell lines. Values represent the mean of 3 replicates ± standard deviation (***$P < .005$, Student *t* test). Two independent experiments were performed. (*C*) Immunoblotting with anti-PARP1 48 hours after transfection of HCT116 and RKO cell lines with SCR, miR-28-5p, or miR-28-3p. Graphic represents the ratio between cleavage and total PARP1 form. miR-28-5p, but not miR-28-3p, increased PARP1 cleavage form. (*D*) Fluorescent-activated cell sorting analysis 48 hours post-transfection with SCR, miR-28-5p, or miR-28-3p. Representative experiment was performed in duplicate; mean ± standard deviation (*$P < .05$, Student *t* test). Two independent experiments were performed.

samples (miR-28-5p, $P < .005$; miR-28-3p, $P < .005$) (Supplementary Figure 3). In order to confirm these results, we used a second independent set of CRC samples. In 23 paired samples of tumors and adjacent normal tissue, we also found that both miRNAs were downregulated (miR-28-5p, $P < .001$; miR-28-3p, $P < .001$) (Figure 1*C*). Values of expression are presented in Supplementary Tables 2 and 3.

miR-28-5p, but Not miR-28-3p, Significantly Suppresses Proliferation and Induces Apoptosis and G1 Arrest in CRC Cells

To elucidate the roles of miR-28-5p and miR-28-3p in CRC tumorigenesis, HCT116 and RKO CRC cell lines (endogenous miR-28 expression levels of colon cell lines are shown in Supplementary Figure 4) were transfected with SCR, pre-miR-28-5p, or pre-miR-28-3p. Expression of miRNAs was confirmed by quantitative real-time PCR (Supplementary Figure 5). In both cell lines, we found that cells overexpressing miR-28-5p grew significantly less ($P < .005$) than did cells transfected with control or miR-28-3p (Figure 2*A* and *B*). This result was also confirmed in the HCT116 and RKO cell lines using the 3-(4,5-dmethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (Supplementary Figure 6*A* and *B*). In contrast, in both cell lines overexpressing miR-28-3p, there were no statistically significant differences at any time (HCT116, $P = .25$; RKO, $P = .81$) compared with cells transfected with control (Figure 2*A* and *B*). Therefore, the in vitro results suggest that miR-28-5p, but not miR-28-3p, has a biological effect on proliferation.

We then explored the possibility that the effect of miR-28-5p on proliferation could be due to an increase in

apoptosis or to defects in the cell cycle. To test whether miR-28-5p had an effect on apoptosis, we measured poly-(adenosine diphosphate-ribose) polymerase 1 (PARP1) protein, which is specifically cleaved by caspases and promotes apoptosis. PARP1 cleavage forms are one of the most reliable apoptotic markers.[13,14] Cells transfected with pre-miR-28-5p expressed 2.2 and 1.8 times more cleaved-PARP1 form (relative to total-PARP1 form) than did cells transfected with control in the HCT116 and RKO cell lines, respectively (Figure 2*C*). In agreement with the results of the proliferation assays, cells transfected with miR-28-3p presented a PARP1 cleaved to total form ratio similar to the control (Figure 2*C*). In addition, our results were confirmed by caspases 3/7, 8, and 9 activities, which were all higher in miR-28-5p–transfected cells than in the SCR-transfected cells (Supplementary Figure 6*C*). In order to analyze possible differences in the cell cycle, the HCT116 cell line was transfected with either SCR, miR-28-5p, or miR-28-3p and analyzed by fluorescent-activated cell sorting. Compared with the control, cells transfected with miR-28-5p had a significantly higher percentage of cells in G1 phase and a significantly lower percentage of cells in S phase, suggesting that miR-28-5p causes G1 arrest ($P < .05$) (Figure 2*D*). Despite being concomitantly transcribed and being part of the same RNA stem-loop hairpin, these data suggest that miR-28-5p has a tumor-suppressive role in CRC and that miR-28-3p does not have the same biologic role.

miR-28 Disrupts Tumor Growth In Vivo

Because our in vitro studies indicated that miR-28-5p acts as a tumor suppressor in CRC, we analyzed the overall effect of miR-28 in vivo. For that purpose, we generated stable clones overexpressing miR-28, and expression of miR-28-5p and miR-28-3p was verified by quantitative real-time PCR (Supplementary Figure 7). HCT116 colon cancer cells stably transfected with pBABE-empty or pBABE-miR-28 were subcutaneously injected into the left and right flanks of each mouse, respectively (n = 9). Both cell lines were injected into the same mice to decrease inter-mouse variability. Tumors derived from the HCT116 stably expressing pBABE-miR-28 cells grew much slower than did tumors derived from the HCT116 stably expressing pBABE-empty cells (Figure 3*A*). Accordingly, final tumor volume in pBABE-miR-28 tumors was significantly reduced ($P < .01$) compared with pBABE-empty tumors (Figure 3*B* and *C*). miR-28 expression levels were confirmed in these tumors. In pBABE-miR-28 tumors, miR-28-5p and miR-28-3p were increased ($P < .01$) when compared with pBABE-empty tumors (Figure 3*D*). In conclusion, this xenograft experiment revealed that expression of miR-28 disrupts tumor growth in vivo.

Opposite Effects of miR-28-5p and miR-28-3p in Cell Migration and Invasion

To better understand the biological importance of miR-28-5p and miR-28-3p in CRC, we explored whether these miRNAs could be involved in colon cancer metastasis. To evaluate the migratory capacity of HCT116 cells expressing either miRNA, we used Transwell cell migration assays. Overexpression of miR-28-5p led to a significant reduction in cell migration ($P < .01$), whereas overexpression of miR-28-3p led to a significant increase ($P < .05$) in cell migration compared with the control (Figure 4). The same result was obtained when using SW480 transfected cells (miR-28-5p, $P < 0.05$; miR-28-3p; $P < .01$) (Supplementary Figure 8). To determine whether both miR-28-5p and miR-28-3p also played a role in invasion, we used Transwell chambers coated with Matrigel. HCT116 cells expressing miR-28-5p had a reduction in invasiveness ($P < .05$), whereas cells expressing miR-28-3p had an increase in invasiveness ($P < .01$) compared with the control (Figure 4). Although no statistically significant differences were obtained for SW480 cell line, the same trend was observed—miR-28-5p overexpressing cells are less invasive and miR-28-3p are more invasive than control (miR-28-5p, $P = .25$; miR-28-3p, $P = .12$) (Supplementary Figure 8). The effect of miR-28-3p, which showed a growth rate similar to the control, on migration and invasion appears to be independent of cell growth. Therefore, although both miRNAs are down-regulated in CRC, they play different roles in the migration phenotype.

miR-28 Increases Metastasis In Vivo

As miR-28-5p and miR-28-3p exert opposite effects on migration and invasion in vitro but are transcribed concomitantly in cells, we investigated the effect of global miR-28 expression on metastasis in vivo. For this purpose, we intravenously injected mice with pBABE-empty or pBABE-miR-28 cells. After 35 days, the mice were sacrificed. At necropsy, tumors were found in the liver, kidney, lung, and spinal cord. We found an increased number of mice with metastases in all tumor sites in the pBABE-miR-28 group compared with the pBABE-empty group (Figure 5*A*). In particular, metastases in the liver and lung were found at a statistically significant higher frequency in the pBABE-miR-28 group than were in the pBABE-empty group ($P < .05$). Examples of tumor metastases from the 3 most frequent locations—liver, kidney, and lung—are presented with H&E staining and anti–green fluorescent protein labeling (Figure 5*B*). In addition, the number of tumors in liver and kidney was higher in the pBABE-miR-28 group than in pBABE-empty (Figure 5*C*). In particular, in the pBABE-miR-28 group, 6 mice presented liver tumors with a mean of 1.5 ± 0.8 tumors per mice, and in the pBABE-empty group, there was only 1 mouse that developed only 1 liver tumor. Regarding the kidney, in the pBABE-miR-28 group, 10 mice presented kidney tumors, with a mean of 14.6 ± 4.2 tumors per mice (considering both kidneys), and in the pBABE-empty group, 6 mice developed kidney tumors, with an average of 6 ± 4.2 tumors per mouse ($P < .005$). An example of the tumors can be visualized in Figure 5*D*. Although miR-28-5p and miR-28-3p had contrasting effects on migration and invasion in vitro, and although in vivo sub-

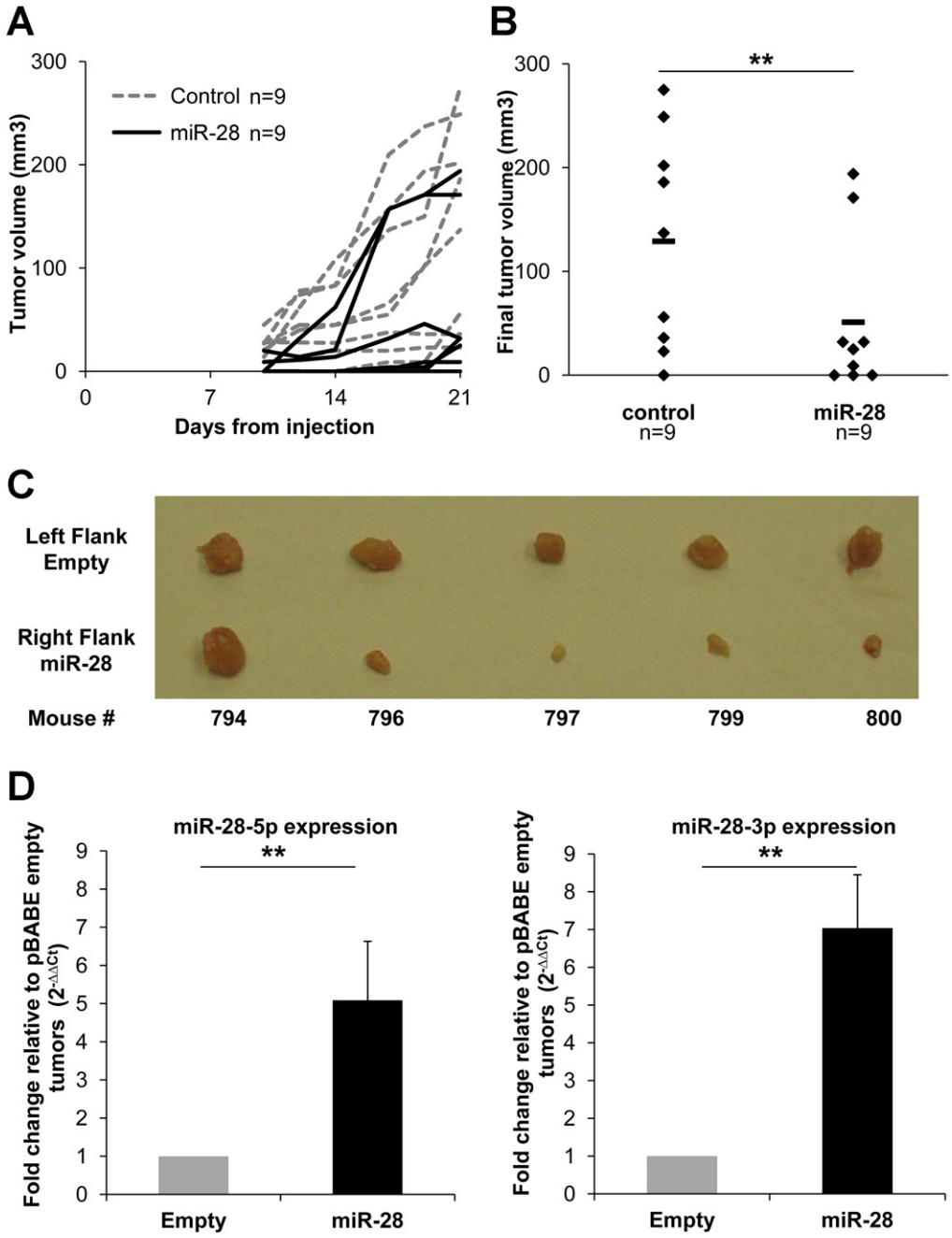

Figure 3. miR-28 decreases tumor volume in mice xenografts. (*A*, *B*) HCT116-pBABE-empty (control) and HCT116-pBABE-miR-28 (stably expressing miR-28) were subcutaneously injected in the left and right flanks of 9 mice, and tumor volume was measured during the (*A*) course of the experiment and (*B*) at the end of the experiment (21 days post inoculation). Tumor volumes in the HCT116-pBABE-miR-28 group were lower than those in the HCT116-pBABE-empty group (**$P < .01$, Student *t* test). (*C*) Photographs show tumors excised from 5 mice in each group. (*D*) Quantitative real-time PCR analysis shows miR-28-5p and miR-28-3p expression in the tumors extracted from the mice (mean ± standard deviation) (**$P < .01$, Student *t* test).

cutaneous tumorigenesis appeared to correlate with the growth-inhibiting effects of miR-28-5p, the overall in vivo results of the metastasis experiments resembled the effects caused by miR-28-3p, indicating that this miRNA may have a predominant effect on metastasis.

miR-28-5p and miR-28-3p Targets

To identify miR-28-5p and miR-28-3p targets that could be involved in the biological effects caused by these miRNAs, we first used an in silico approach. By selecting the targets predicted to be regulated by miR-28-5p or miR-28-3p in PITA, TargetScan, and miRanda programs simultaneously, we found 5784 mRNAs. Of these mRNAs, 2629 were predicted to be a target of miR-28-5p but not miR-28-3p; 1305 were predicted to be a target of miR-28-3p but not miR-28-5p; and 925 were predicted to be targets of both miRNAs. To narrow the list of potential targets, we focused on those that have been described as up-regulated in colon cancer (given that miR-28 is down-regulated) and have been reported to be involved in the biological functions investigated here. Therefore, we searched for miR-28-5p targets involved in proliferation and miR-28-3p targets involved in metastasis, and we considered targets that were predicted by at least 2 programs. In this way, we identified CCND1, HOXB3, and NM23-H1.

We first used immunoblotting to detect changes at the protein level for several predicted targets of interest in cells transfected with SCR, miR-28-5p, or miR-28-3p. We

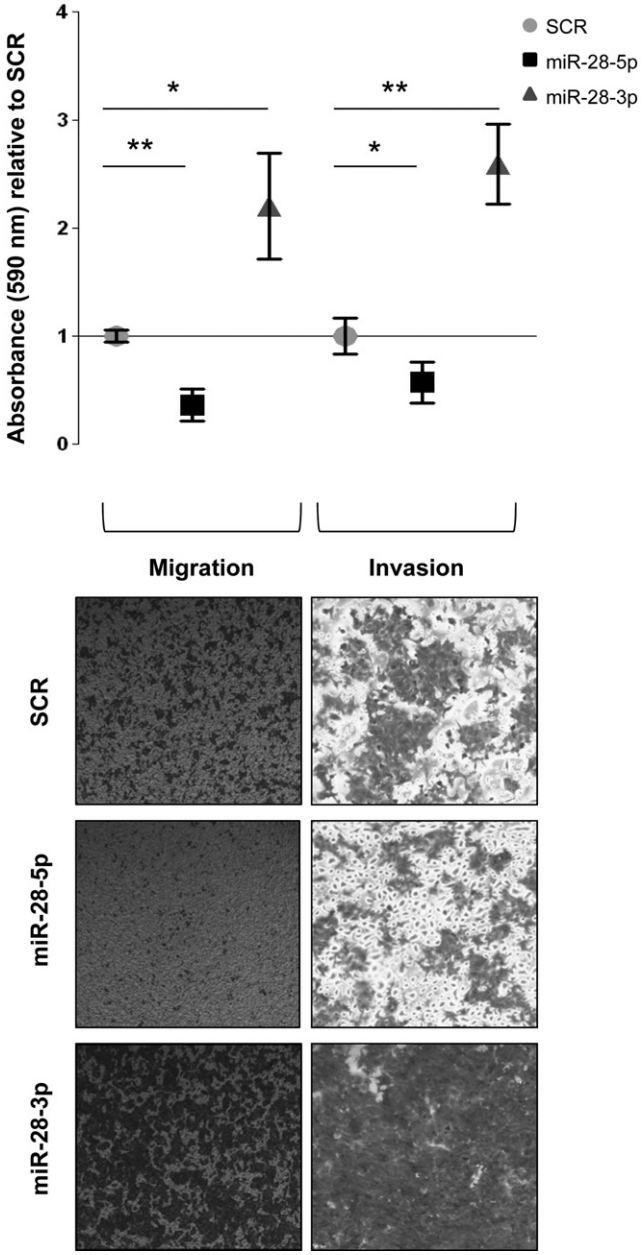

Figure 4. Effect of mir-28-5p and miR-28-3p on migration and invasion in vitro. Absorbance was measured for cells on the bottom of noncoated and Matrigel-coated Transwell chambers at 24 hours (for migration) and 48 hours (for invasion) after HCT116 cells expressing miR-28-5p or miR-28-3p were plated. Results are shown relative to SCR. A representative experiment is shown. Mean of triplicates ± standard deviation is shown ($^{*}P < .05$; $^{**}P < .01$, Student *t* test). Microscopy images (×50) show the migratory and invasive cells on Transwell assays.

found a 51% reduction in the level of cyclin D1 (encoded by the *CCND1* gene) in cells in which miR-28-5p was restored. On the contrary, no differences in cyclin D1 levels were detected in miR-28-3p–expressing cells compared with SCR-transfected cells (Figure 6*A*). We also found that *HOXB3* was a target of miR-28-5p because this miRNA reduced HoxB3 protein expression by 35% (Figure 6*B*). Regarding miR-28-3p, we found that the protein Nm23-H1 was down-regulated by 52% in cells expressing miR-28-3p (Figure 6*C*).

To determine whether the effect on these targets was caused by direct binding of the miRNAs or by an indirect effect, we cloned the predicted mRNA binding sites (Figure 6*D* and *E*; Supplementary Figure 9) downstream of the modified coding region of firefly luciferase in pGL3 reporter vector. We found that miR-28-5p significantly reduced luciferase activity in the *HOXB3* reporter construct by 38% ($P < .01$) (Figure 6*D*). Also, miR-28-3p reduced luciferase activity in the *NM23-H1* reporter construct by 34% ($P < .01$) (Figure 6*E*), and no significant differences were found when cells were cotransfected with miR-28-5p and the *NM23-H1* construct (Supplementary Figure 10). To confirm this specific interaction, we mutated the miRNA-binding sites, and the luciferase activity for the PGL3-*HOXB3* and PGL3-*NM23-H1* constructs was restored to the same levels as the control. Regarding *CCND1*, although we found a significant decrease in luciferase activity in miR-28-5p–transfected cells, the binding site mutation did not fully restore the luciferase activity to the control level (Supplementary Figure 9). In summary, we found that miR-28-5p targeted cyclin D1 and HoxB3 and that miR-28-3p targeted Nm23-H1; this could explain, at least in part, the biological effects observed.

Discussion

In the present study, we analyzed 2 independent sets of human CRC samples, for a total of 108 (47 paired with normal tissue), and found significant down-regulation of both mature miR-28 forms. Our study is the first to show down-regulation of miR-28 in cancer. In the literature, only 1 study extensively analyzed miR-28 function in cancer, namely in myeloproliferative neoplasms. Girardot et al identified miR-28 overexpression in platelets of BCR-ABL–negative myeloproliferative neoplasm patients and found myeloproliferative leukemia virus oncogene to be the main target, which is important for megakaryocyte differentiation.[15] In normal colon tissue, in situ hybridization shows that miR-28-5p and miR-28-3p are predominantly expressed in epithelial cells (Supplementary Figure 11). In addition, a couple of profiling studies showed miR-28 up-regulation in renal cell carcinoma[16] and during glioma progression.[17] It is well established that miRNAs can function as either tumor suppressors or oncogenes, depending on the tumor tissue and the cell type.[5] Therefore, when studying miRNAs, it is essential to take into consideration the cellular context.[5,18] One of the best examples is miR-125a/b, which has been shown to be down-regulated in glioblastoma, breast, prostate, ovarian, and non−small cell lung cancer, but up-regulated in myelodysplastic syndrome and acute myeloid leukemia patients with t(2;11)(p21;q23) and in urothelial carcinoma.[18,19] Noteworthy, miRNA variation levels between normal tissue and tumors of <50% are reported frequently, and the Volinia et al study, which represents the largest miRNA profiling study reported so far, shows as highly significant consistent variations of <20%.[20]

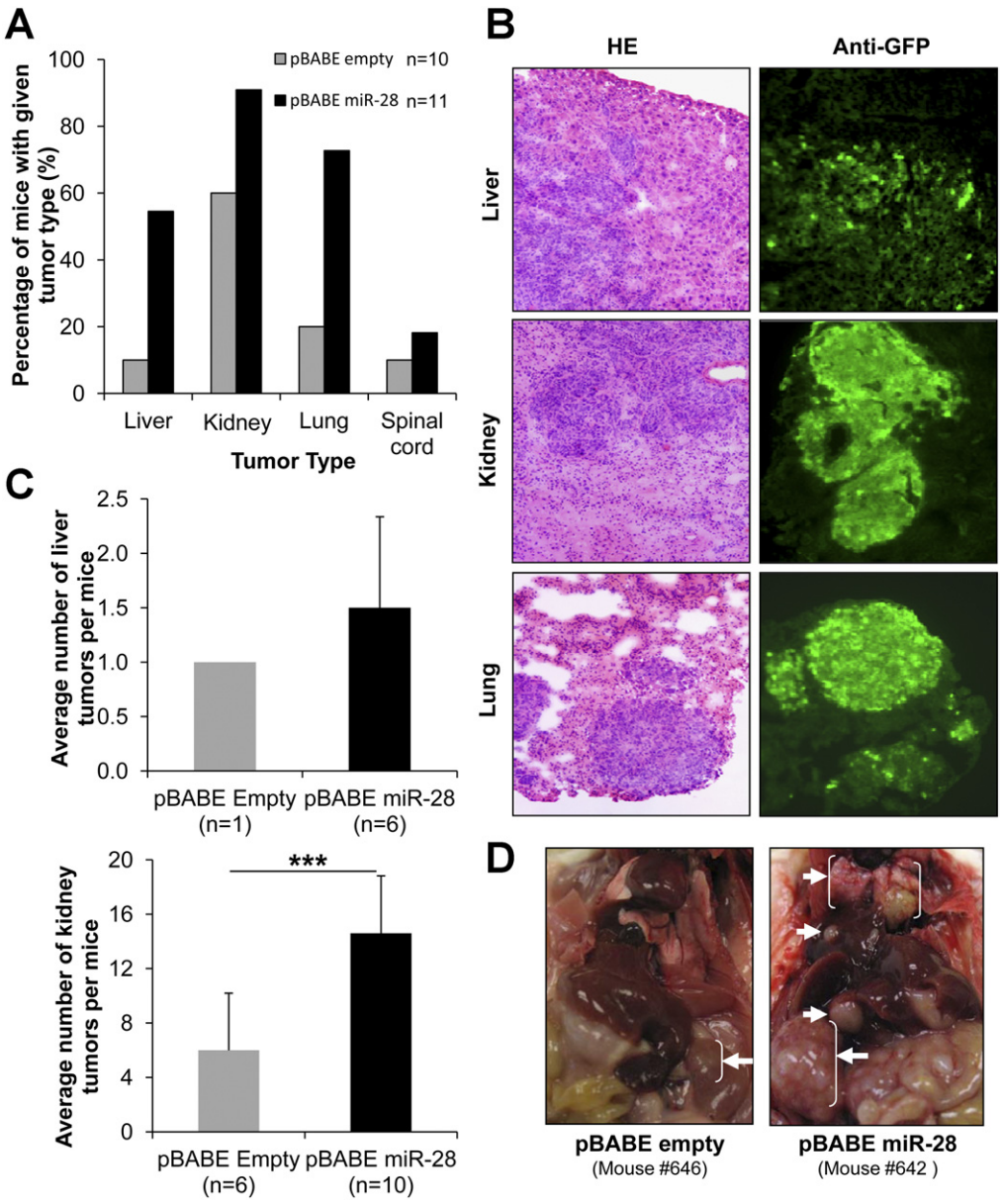

Figure 5. miR-28 increases metastasis in vivo. (*A*, *B*) HCT116-pBABE-empty (control) and HCT116-pBABE-miR-28 (stably expressing miR-28) were injected in the vein tail of mice. (*A*) Thirty-five days postinjection metastases were detected in the liver, kidney, lung, and spinal cord. The percentage of mice with metastases in these organs was consistently higher in miR-28–expressing tumors than in the control. (*B*) Microscopy images (×100) show H&E (HE) and anti–green fluorescent protein (GFP) immunohistochemical staining for liver, kidney, and lung metastatic tumors. (*C*) Number of tumors observed within the liver and kidneys. ***$P < .005$. (*D*) Photographs of HCT116-pBABE-empty (*left panel*) and HCT116-pBABE-miR-28 (*right panel*) mice show the sites with metastasis (*white arrows*) found in >30% of each group of mice.

As down-regulation of miR-28-5p and miR-28-3p had never been described before, we analyzed their roles in CRC in detail. This study provides evidence that strand-specific 5p and 3p miRNAs have distinct functions (Figure 6*F*). Concordantly with the role of a tumor-suppressor gene, miR-28-5p suppressed cell proliferation, causing apoptosis and G1 arrest in the cell cycle; however, miR-28-3p had no effect on proliferation in vitro. Therefore, the overall effect in vivo was, as expected, a significant decrease in tumor volume. In contrast, miR-28-5p and miR-28-3p caused opposite effects in migration and invasion in vitro. The miR-28–injected mice developed more metastases than did the control mice, which is in agreement with the in vitro effect observed for miR-28-3p–overexpressing cells. To our knowledge, only 2 studies have addressed the distinct roles of 5p and 3p strands, but none of them have investigated the in vivo effect or the distinct targeting mechanisms in detail. These studies showed the different effects of miR-125a-3p and miR-125a-5p in lung cancer cells[19] and miR-34c-3p and miR-34c-5p in the cervical tumor cell line SiHa.[21]

Recently, Yang et al identified the *erythroid 2–related factor 2* as a target of miR-28 in breast cancer.[22] To understand the underlying mechanisms of miR-28, we searched for miRNA targets (Figure 6*F*). Cyclin D1, encoded by the *CCND1* gene, is a well-known oncogene that is overexpressed in several types of tumors, including CRC.[23] This protein is a key player in cell-cycle regulation, in particular in the G1–S phase transition,[24,25] and its inhibition reduces growth and tumorigenicity in human colon cancer cells.[26] We found that miR-28-5p, but not miR-28-3p, targets cyclin D1. This is in agreement with the biological functions of miR-28-5p, as only miR-28-5p and not miR-28-3p caused G1 arrest. Although cyclin D1 protein levels were decreased in miR-28-5p–transfected cells, it remains to be determined whether this is a consequence of a direct miR::mRNA interaction or

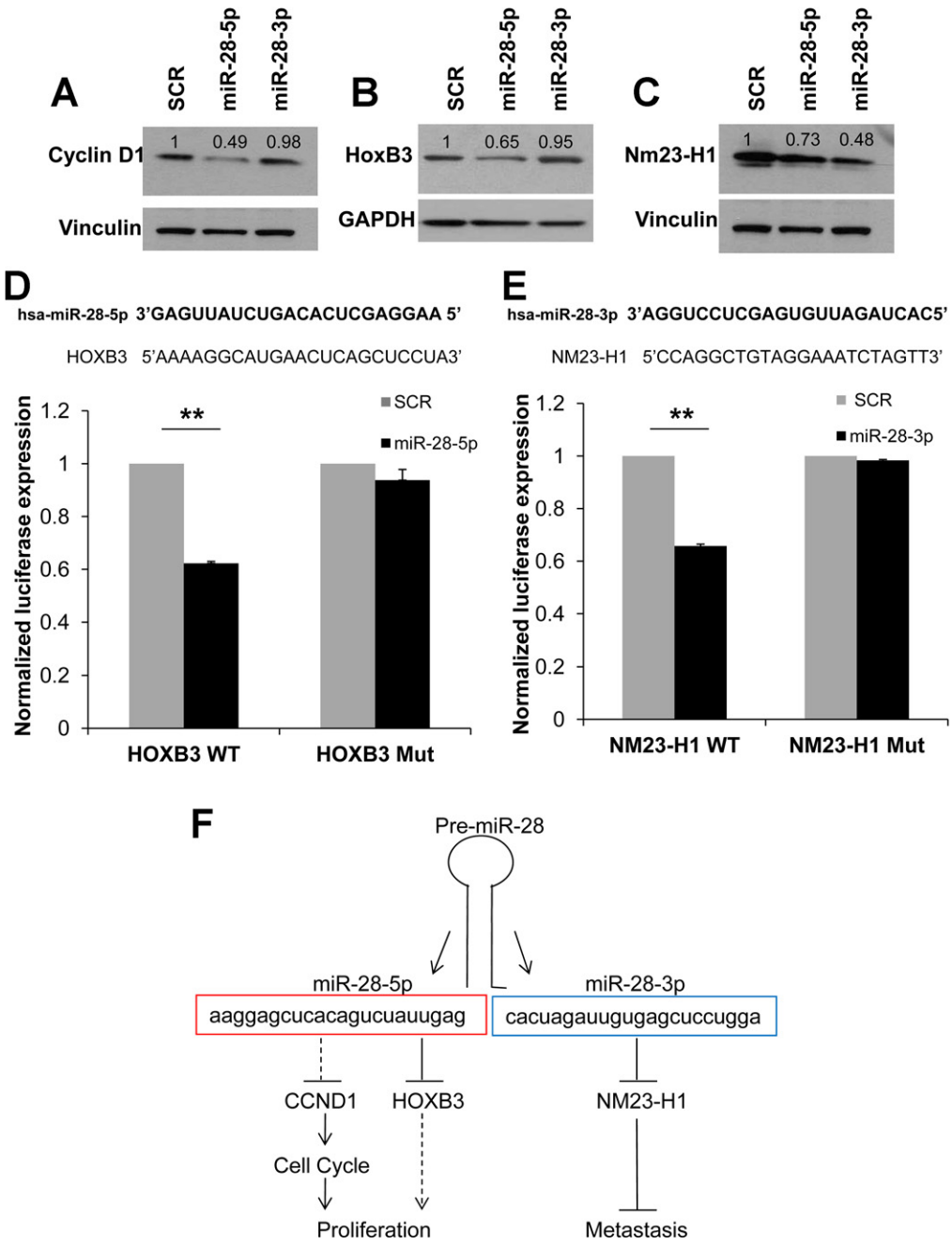

Figure 6. miR-28-5p targets cyclin D1 and HoxB3, and miR-28-3p targets Nm23-H1. Western blot analysis shows (*A*) cyclin D1, (*B*) HoxB3, and (*C*) Nm23-H1 expression in scrambled, miR-28-5p, and miR-28-3p transfected HCT116 cells. Expression levels were normalized for vinculin or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) protein levels and were compared with the SCR transfection (=1). (*D*, *E*) The predicted miRNA::mRNA interaction sites are shown in the *top panels*. The *bottom panels* show luciferase activity for the predicted interaction sites (*D*) PGL3-*HOXB3*-WT constructs cotransfected with SCR (n = 1) or miR-28-5p and (*E*) PGL3-*NM23-H1*-WT construct cotransfected with SCR (n = 1) or miR-28-3p. The same experiment was also performed using constructs with a mutated interaction site—PGL3-*HOXB3*-Mut and PGL3-*NM23-H1*-Mut. Values represent the mean ± standard deviation of 2 independent experiments performed in 4 replicates ($^{**}P < .01$, Student *t* test). (*F*) The proposed mechanism for miR-28-5p and -3p function in CRC is shown.

an indirect effect through miR-28-5p targeting of other mRNAs in pathways where cyclin D1 is involved. The miR-28-5p::*CCND1* binding site predicted in silico showed a slight luciferase reduction that was not abrogated by the binding site mutation, showing that at least in this site there is no direct interaction. However, and although not predicted by our program's analysis, we do not exclude the possibility that other miR-28-5p::*CCND1* binding sites might exist. In addition, we also found *HOXB3* to be a target of miR-28-5p. *HOXB3* has been described as being significantly overexpressed in colon cancer.[27] Although the role of *HOXB3* in colon cancer has not been explored, Palakurthy et al described a mechanism by which *HOXB3* exerts it oncogenic role, showing that it is essential for epigenetic silencing of the tumor-suppressor *RASSF1A*,[28] the promoter of which is hypermethylated in colon tumors.[29] These authors also demonstrate in a lung cancer cell line that *HOXB3* increases tumor growth both in vitro and in vivo.[29] In addition, *HOXB3* has been demonstrated to regulate cellular proliferation of hematopoietic stem cells[30] and of Rat-1 cell line.[31] The interaction between miR-28-5p and *HOXB3* occurs through a direct binding as demonstrated by the luciferase assay results. Our data demonstrate that, in vivo, miR-28 promotes metastasis and that, in vitro, miR-28-3p induces migration and invasion. As miR-28 was reduced in the tumors, we looked for an antimetastatic mRNA as a target, which would suppress metastasis without affecting tumor growth.[32] Interestingly, we found that miR-28-3p has the capacity of regulating *NM23-H1*, the first metastasis-suppressor gene identified.[33–35] Remarkably, it has been previously

reported that this gene is overexpressed in colon carcinoma cells, especially in the early stages, and that it limits the invasive potential of human cancer cells without having an effect on proliferation.[36] In addition, *NM23-H1* inhibits liver metastases of colon.[37]

In the future, prospective studies should be performed to address clinical correlations and systematic experiments should be conducted to identify all potential targets that can explain the distinct biological effects.

In conclusion, this is the first study to report downregulation of miR-28 in human tumorigenesis. In CRC, miR-28 suppresses proliferation but activates metastasis; this is a consequence of the distinct roles of the miR-28 hairpin RNA products, miR-28-5p and miR-28-3p. Such information has direct consequences for the design of miRNA gene therapy trials. The manipulation of the expression of specific miRNAs by using the precursor molecules can produce additional clinical effects due to the transcription of 5p and 3p genes with distinct biological effects.

Supplementary Material

Note: To access the supplementary material accompanying this article, visit the online version of *Gastroenterology* at www.gastrojournal.org, and at doi:10.1053/j.gastro.2011.12.047.

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Conflicts of interest

The authors disclose no conflicts.

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Supplementary Methods

Microsatellite Analysis

Microsatellite analysis was performed on DNA extracted from frozen tissue samples by a standard phenol-chloroform procedure. MSI was evaluated with a fluorescence-based PCR method using the 5 markers of the Bethesda panel (ie, D5S346, D17S250, D2S123, BAT25, and BAT26) plus BAT40. Analysis of PCR products was done with an automated DNA sequencer. Tumors were classified as MSS, MSI-L, and MSI-H according to the guidelines of the International Workshop of Bethesda.[1]

RNA and Protein Extraction

RNA was isolated using Trizol reagent (Invitrogen), according to manufacturer's instructions. RNA quantity and purity was assessed with NanoDrop ND-1000 (Thermo Fisher Scientific, Wilmington, DE). RNA integrity was analyzed by gel electrophoresis. RNA samples were denaturated at 70°C for 5 minutes, immediately placed on ice, and loaded on an agarose gel stained with ethidium bromide. Intensity of the 18S and 28S bands was examined.

Total protein extracts were prepared in ice-cold lysis buffer (0.5% Nonidet P-40, 250 mM sodium chloride, 50 mM HEPES, 5 mM EDTA, and 0.5 mM ethylene glycol-bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid) containing phosphatase inhibitor cocktail 2 (Sigma-Aldrich, St Louis, MO), protease inhibitor (Clontech, Mountain View, CA), and dithiothreitol (Invitrogen).

Reverse Transcription Quantitative Real-Time PCR

miRNA expression was evaluated using TaqMan miRNA assays (Applied Biosystems). Briefly, complementary DNA was synthesized using RNA as a template, gene-specific stem-loop Reverse Transcription primer, and the TaqMan microRNA reverse-transcription kit (Applied Biosystems). Quantitative real-time PCR was carried out in a CFX384 real-time system (Bio-Rad, Hercules, CA) using complementary DNA, TaqMan probe, and TaqMan universal PCR master mix (Applied Biosystems). Experiments were performed in duplicate and normalized to small nuclear RNA U6, which was used as an internal control. Relative expression levels were calculated using the comparative cycle threshold method. Stability of the reference gene between samples was analyzed. PCR efficiency was determined using the formula: Efficiency $= 10^{-1/\text{slope}} - 1$.

Cell Culture, STR DNA Fingerprinting, and miRNA Mimics Transfection

Human CRC HCT116, RKO, and SW480 cell lines (purchased from American Type Culture Collection, Manassas, VA) were grown as suggested by the supplier. Cells were cultured at 37°C in 5% CO_2.

All cell lines used in this study were validated by STR DNA fingerprinting using the AmpFℓSTR Identifiler kit, according to manufacturer instructions (Applied Biosystems). The STR profiles were compared with known ATCC fingerprints (ATCC.org), to the Cell Line Integrated Molecular Authentication database version 0.1.200808 (http://bioinformatics.istge.it/clima/),[2] and to the MD Anderson fingerprint database. STR profiles of HCT116, RKO, and SW480 cell lines matched known DNA fingerprints and were unique.

Pre-miRNA miRNA precursor molecules for hsa-miR-28-5p and hsa-miR-28-3p and pre-miR miRNA precursor SCR #2 were purchased from Ambion (Austin, TX). Transfections were performed using 50 nM miRNA specific-strand precursor molecules or control and Lipofectamine 2000 reagent (Invitrogen), according to manufacturer's instructions. RNA and proteins were collected at 48 hours after transfection. miRNA transfection efficiencies were evaluated by reverse transcription quantitative real-time PCR.

3-(4,5-Dmethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) Assay

We seeded 5×10^3 HCT116 cells transfected with either SCR or miR-28-5p in a 96-well plate in 8 replicates for each condition. At each time point (0, 24, 48, 72, and 96 hours post transfection), the colorimetric reagent was added to the cells. After 2-hour incubation at 37°C, dimethylsulfoxide was added. Proliferation was assessed by measuring absorbance at 580 nm using the SpectraMax Plus^{384} microplate reader (Molecular Devices, Sunnyvale, CA). Experiment was performed 2 times independently.

Apoptosis Quantification

Protein levels of the apoptotic molecular marker PARP1, full-length, and cleavage PARP1 forms were assessed by Western blot analysis using PARP antibody (9542) from Cell Signaling Technology (Danvers, MA) in the HCT116 and RKO cell lines transfected with SCR, miR-28-5p, or miR-28-3p. Relative intensity of bands observed by Western blotting was obtained using ImageJ software (http://imagej.nih.gov/ij/). In addition, caspase 3/7, 8, and 9 activity was measured.

Caspase 3/7, 8, and 9 Activity

Caspase activity was measured using Caspase-Glo 3/7 Assay Systems, Caspase-Glo 8 Assay Systems, and Caspase-Glo 9 Assay Systems (Promega Corporation, Madison, WI) in HCT116 cells transfected with SCR, miR-28-5p, or miR-28-3p. The assay was performed 48 hours post transfection according to manufacturer's instructions, and luminescence was measured in a POLARstar OPTIMA microplate reader (BMG Labtech, Ortenberg, Germany).

Cell-Cycle Analysis by Flow Cytometry

For fluorescent-activated cell sorting analysis, 6×10^5 HCT116 cells transfected with either SCR, miR-28-5p, or miR-28-3p were plated onto 6-well plates. After 48 hours, cells were collected and fixed with 70% ice-cold ethanol. Cells were stained with a solution containing 0.05 mg/mL propidium iodide (Sigma-Aldrich) and 0.1 mg/mL RNase A (Roche, Indianapolis, IN) in phosphate-buffered saline. Cell-cycle analysis was performed in a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA). Results were analyzed using ModFit LT software.

In Vitro Cell Migration and Invasion Assays

After 24- or 48-hour incubation (for migration and invasion assay, respectively) at 37°C with 5% CO_2, cells were fixed with paraformaldehyde (USB Corporation, Cleveland, OH). Cells on the upper surface of the chamber (nonmigratory cells) were removed using cotton swabs, and cells on the bottom surface (migratory cells) were stained with crystal violet in 20% methanol for 20 minutes. Finally, 30% acetic acid was added to dissolve the crystal violet and absorbance was measured in a SpectraMax Plus^{384} spectrophotometer (Molecular Devices) at 590 nm.

Establishment of miR-28-Expressing Cell Line: Cell Transduction With Retroviral Vector

A PCR fragment of 483 nt that included the human miR-28 precursor and flanking sequences was amplified using primers with BamHI and EcoRI endonucleases restriction sites (Supplementary Table 4). pBABE-puro retroviral plasmid and miR-28-containing fragment were digested with BamHI and EcoRI enzymes and ligated using T4 DNA ligase (New England Biolabs, Ipswich, MA). Constructs were checked by direct sequencing. The retroviral plasmid pBABE-miR-28 was transiently transfected together with pVSV-G vector into GP2-293 cells using Lipofectamine 2000 reagent (Invitrogen). The retroviral plasmid pBABE-empty was used as a control. Cells were fed with fresh medium the day after transfection. Viral supernatant was collected 3 days after transfection, filtered through 0.45-μm pore, and supplemented with Sequa-brene (Sigma-Aldrich). HCT116 cells, which are known to have metastatic potential,[3] were infected and selected using puromycin. Successful establishment of HCT116-pBABE-miR-28 cell line was verified by reverse transcription quantitative real-time PCR.

Cell Transduction With Lentiviral Vector

As pBABE-puro does not contain green fluorescent protein marker, and to facilitate the detection of the human colon cancer cells in the in vivo studies, HCT116-pBABE-empty and HCT116-pBABE-miR-28 cells were transduced in parallel with empty pRRL-CMV-PGK-GFP-WPRE (Tween) lentiviral vector. Briefly, pTween vector was cotransfected with the packaging vector pCMVDR8.74 and the envelope vector pMD.G into 293FT cells using Lipofectamine 2000 reagent. Forty-eight hours after transfection, supernatant containing the virus was collected, filtered through 0.45-μm pore, and supplemented with Sequa-brene. HCT116-pBABE-empty and HCT116-pBABE-miR-28 were incubated with the viral soup for 45 minutes and centrifuged at 32°C at 1800 rpm, plus another 1 hour and 15 minutes in the incubator at 37°C. Infection efficiency was evaluated by flow cytometry by detecting the percentage of green fluorescent protein–positive cells (>85%).

miRNA Target Prediction

We performed in silico analysis to determine miR-28-5p- and miR-28-3p-predicted targets using an in-house Perl script that scans the databases for the algorithms PITA (http://genie.weizmann.ac.il/pubs/mir07), TargetScan (http://www.targetscan.org), miRanda (http://www.microrna.org), and RNA22 (http://cbcsrv.watson.ibm.com/) for target identification. miR-28 sequence annotation was obtained from the miRBase database (http://www.mirbase.org/) (Supplementary Table 4).

Western Blot Analysis for miRNA Targets

Proteins were collected 48 hours after cells were transfected with SCR, miR-28-5p, or miR-28-3p. Bradford assay was used to measure protein concentration. Proteins were separated by polyacrylamide gel (Bio-Rad) electrophoresis and were transferred to 0.2-μm nitrocellulose membranes (Bio-Rad). The following antibodies were used: anti–cyclin D1 (sc-20044), anti-HoxB3 (sc-28606), and anti-Nm23-H1 (sc-343) all from Santa Cruz Biotechnology (Santa Cruz, CA). Proteins were detected by chemiluminescence. Anti−glyceraldehyde-3-phosphate dehydrogenase from Cell Signaling Technology or anti-vinculin (sc-5573) from Santa Cruz Biotechnology were used as normalizers.

Luciferase Reporter Assays

Fragments of about 200 nt that contained the miR-28-5p and miR-28-3p putative binding sites were amplified by PCR using primers containing the XbaI restriction enzyme site (Supplementary Table 4). PCR products were purified, digested, and directly cloned into the Xbal site of the pGL3 control vector (Promega Corporation, Madison, WI) located downstream of the firefly luciferase reporter gene. The QuikChange II XL site-directed mutagenesis kit (Agilent Technologies, Santa Clara, CA) was used to generate mutations in the miRNA-binding site (Supplementary Table 4).

HCT116 cells were seeded (1×10^5 cells/well) in 24-well plates. After 24 hours, cells were cotransfected with 50 nM SCR, miR-28-5p, or miR-28-3p and 0.4 μg pGL3-putative binding site plasmids or pGL3-mutated putative

binding site plasmids, together with Renilla luciferase construct, which was used as a normalization reference. Transfections were performed in OPTI-MEM I (Invitrogen) using Lipofectamine 2000 reagent. Cells were lysed 48 hours after transfection, and luciferase activity was measured using a dual-luciferase reporter assay system (Promega Corporation) in the veritas microplate luminometer (Turner Biosystems, Sunnyvale, CA). Two independent experiments were performed with 4 replicates each. Normalized relative luciferase activity was calculated by the formula: [firefly luciferase]/[Renilla luciferase] activity. All constructs were confirmed by direct sequencing using an ABI 3730xl DNA analyzer sequencer (Applied Biosystems).

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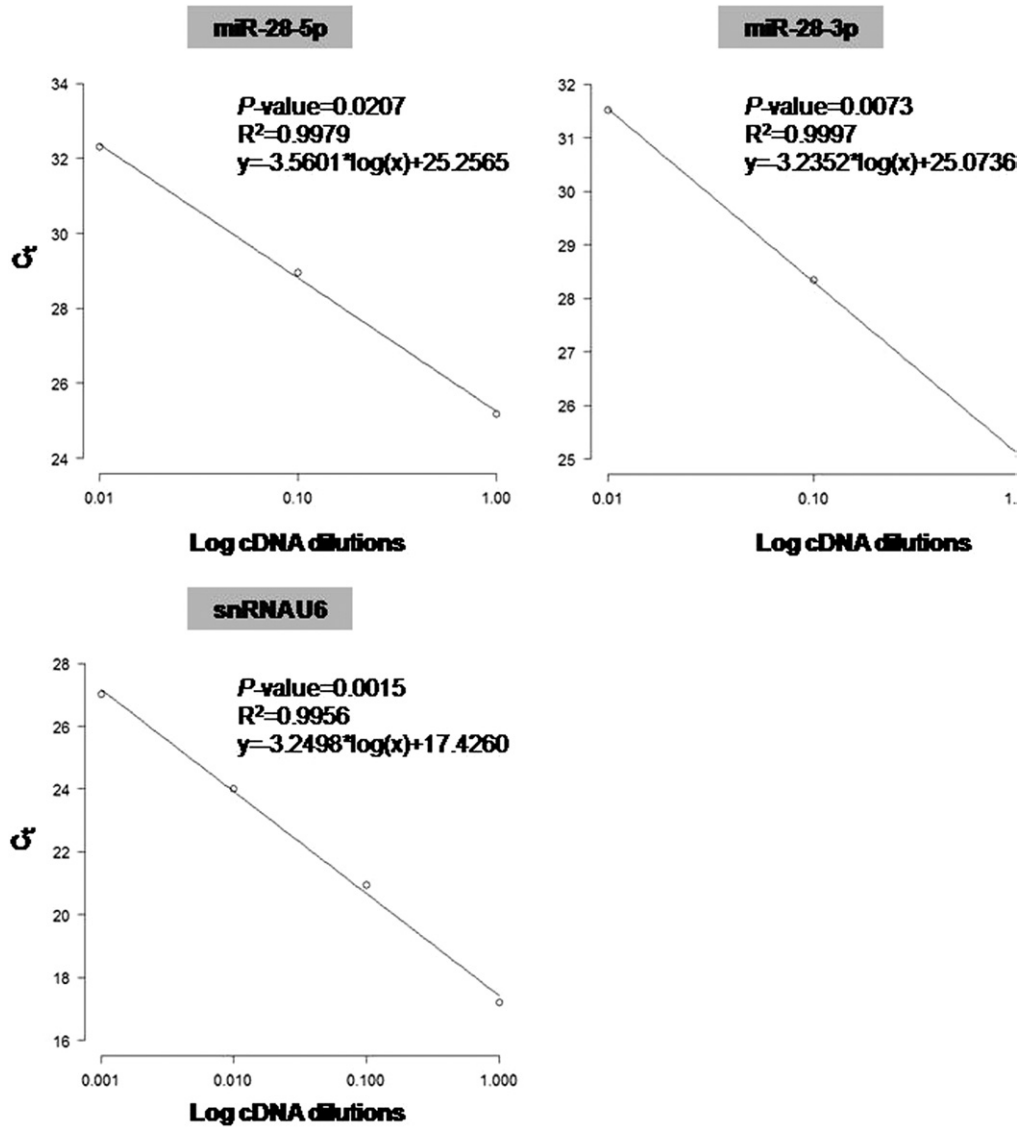
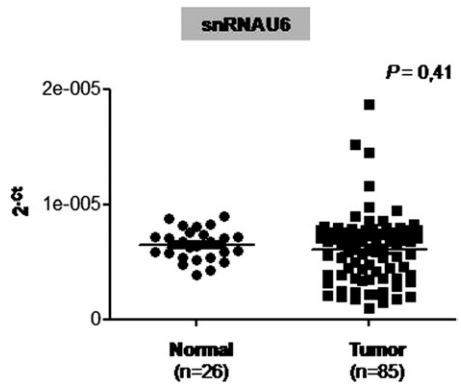
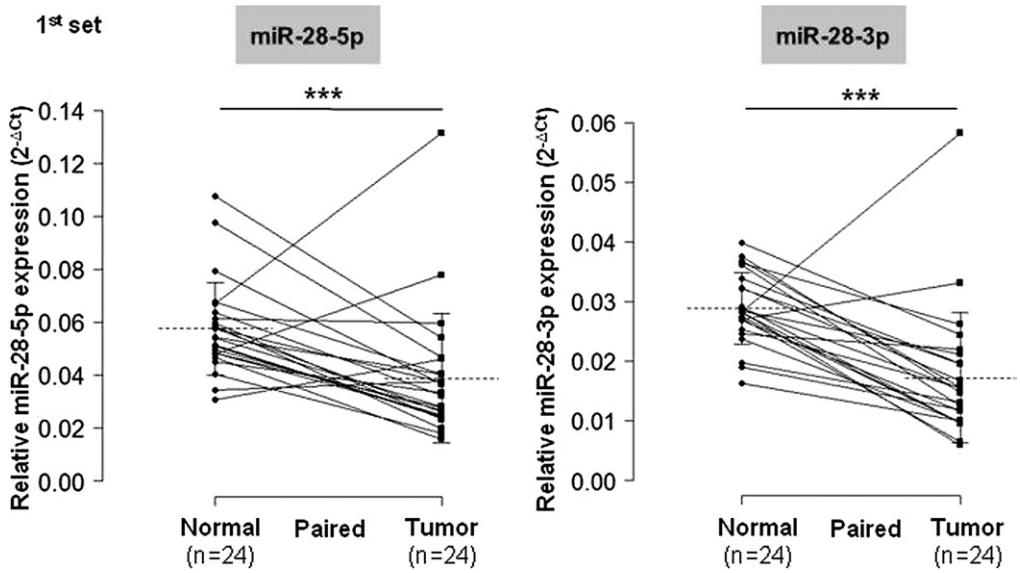


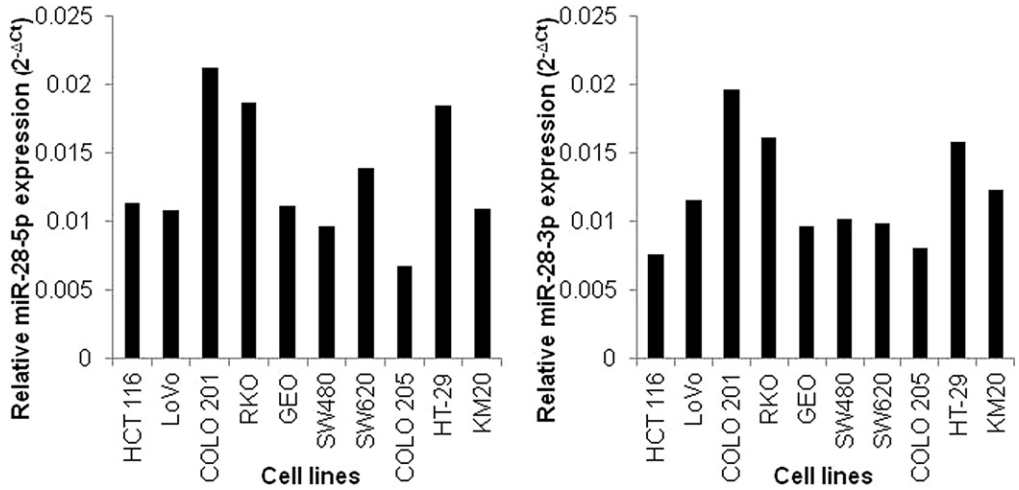
Supplementary Figure 1. Calibration curve determination of Taqman assays for miR-28-5p, miR-28-3p, and small nuclear RNA U6. Serial 10-fold dilutions of complementary DNA were amplified by quantitative real-time PCR. Equation and *P* values were determined using R software.



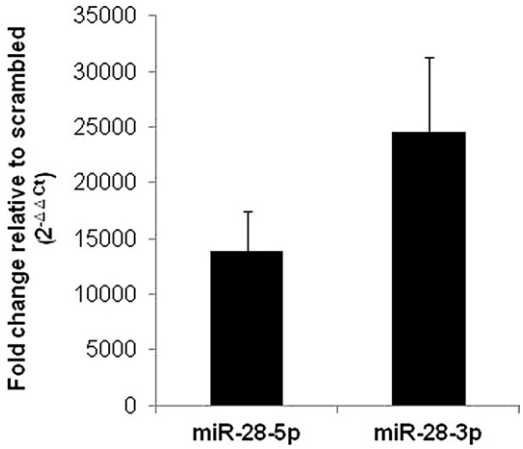
Supplementary Figure 2. Evaluation of the reference gene small nuclear RNA U6 (snRNA U6) variations between samples from normal colon and tumor tissue. There are no differences in small nuclear RNA U6 expression between the 2 groups (P = .41, Mann–Whitney–Wilcoxon test).



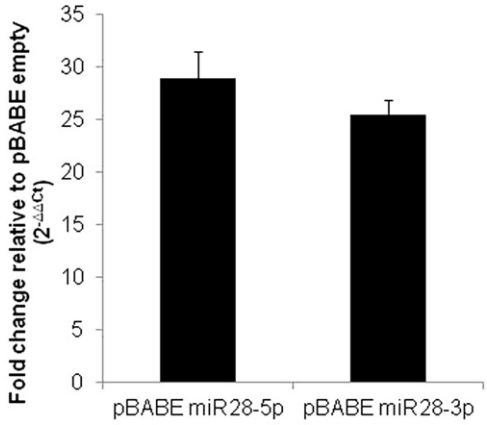
Supplementary Figure 3. Twenty-four normal specimens from the first set of patients were paired with colon cancer tissues from the same patient. All values of miRNA expression levels were normalized by small nuclear RNA U6. Significant differences were ***$P < .005$ using paired *t* test.



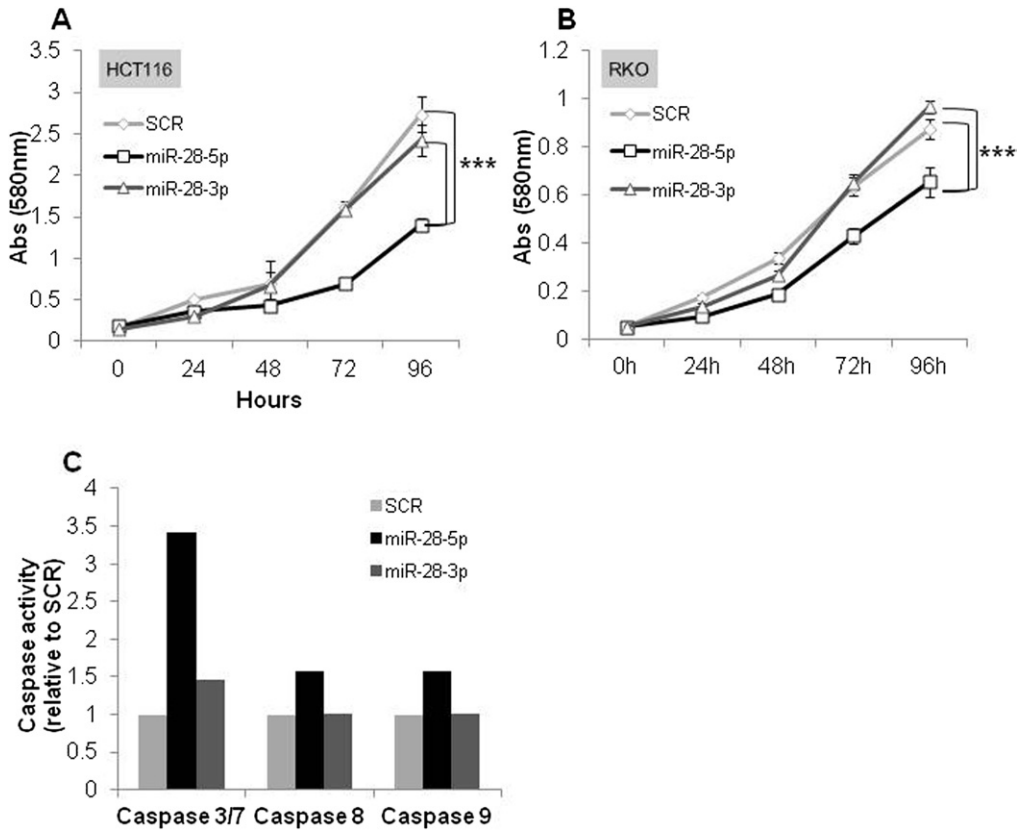
Supplementary Figure 4. Endogenous levels of miR-28-5p and miR-28-3p in 10 colon cancer cell lines. Small nuclear RNA U6 was used as a normalizer.



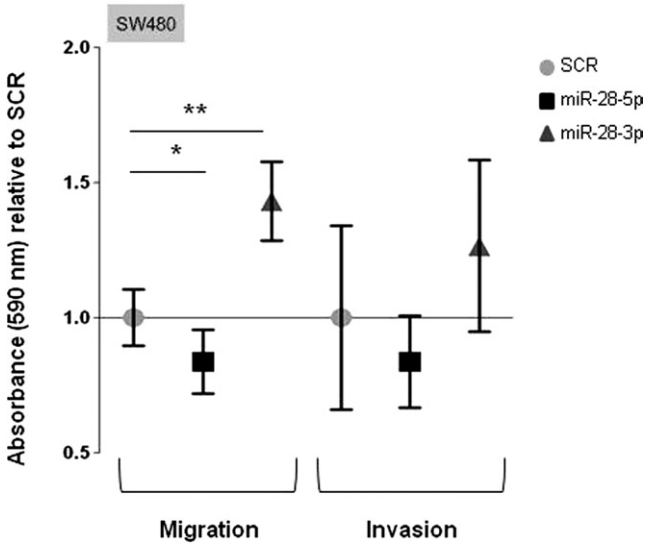
Supplementary Figure 5. miR-28-5p and miR-28-3p levels were measured by quantitative real-time PCR after transient transfection of HCT116 cells with miR-28-5p and miR-28-3p precursors. Values were normalized to small nuclear RNA U6 and are representative of 2 independent experiments. Values shown are relative to negative control.



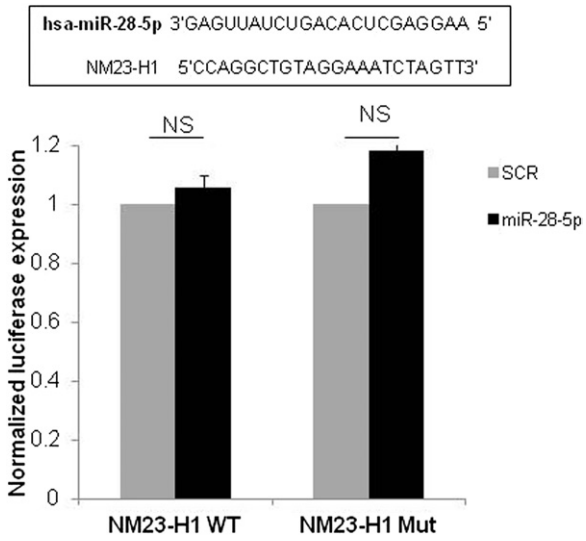
Supplementary Figure 7. miR-28-5p and miR-28-3p levels were measured by quantitative real-time PCR after generating the stable clone pBabe-miR-28 in the HCT116 cell line. Values were normalized to small nuclear RNA U6 and are representative of 2 independent experiments. Values shown are relative to the control pBABE-empty (n = 1).



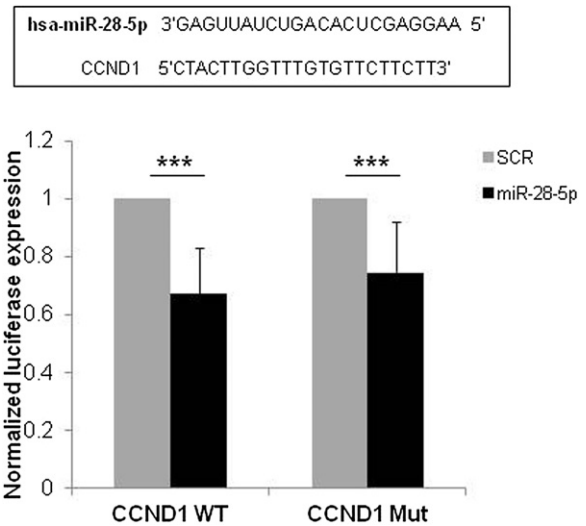
Supplementary Figure 6. 3-(4,5-dmethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide proliferation assay in (*A*) HCT116 and (*B*) RKO cell lines. miR-28-5p, but not miR-28-3p, inhibited cell growth compared with SCR. Values represent the mean ± standard deviation of 8 replicates. (*C*) Caspase activity was measured in the HCT116 cell line 48 hours after transfection with SCR (n = 1), miR-28-5p, or miR-28-3p.



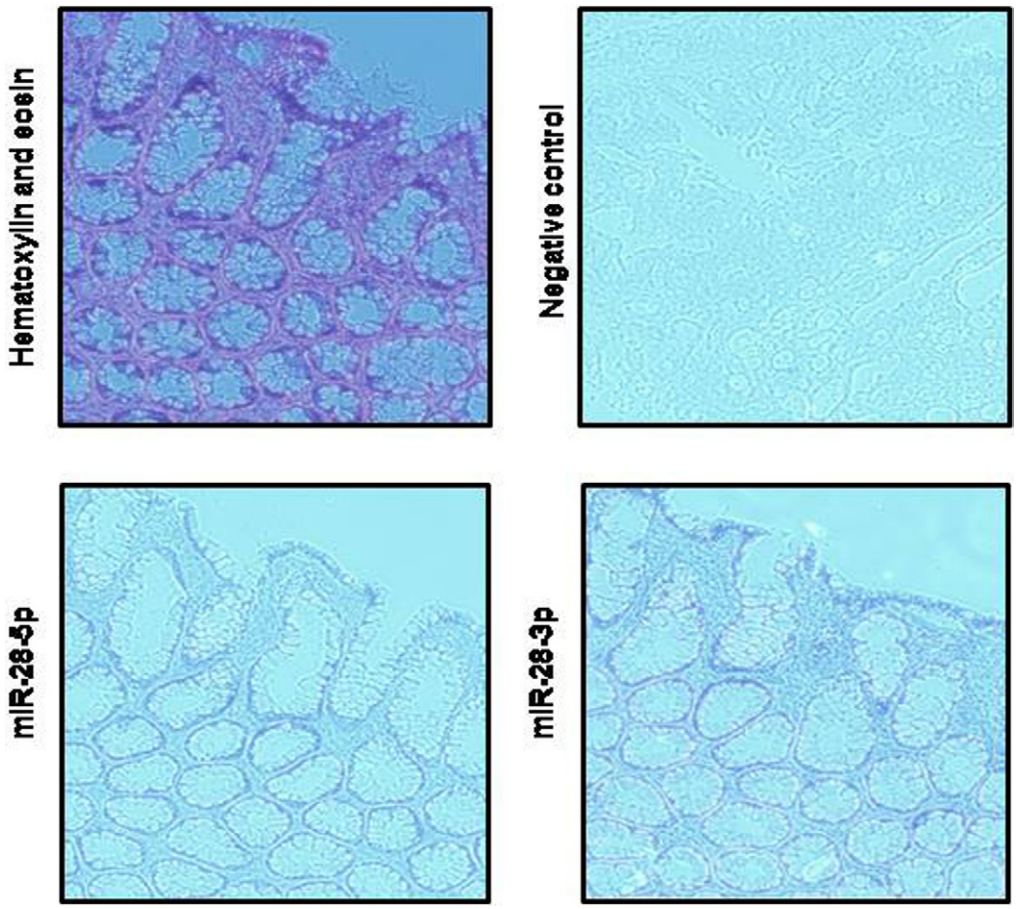
Supplementary Figure 8. Effect of mir-28-5p and miR-28-3p in migration and invasion in vitro in SW480 cell line. Absorbance was measured for cells on the bottom of noncoated and Matrigel-coated Transwell chambers at 24 hours (for migration) and 48 hours (for invasion) after SW480 cells expressing miR-28-5p or miR-28-3p were plated. Results are shown relative to SCR. A representative experiment is shown. Mean of triplicates ± standard deviation is shown (*$P < .05$; **$P < .01$, Student *t* test).



Supplementary Figure 10. Luciferase activity of HCT116 cells cotransfected with SCR (n = 1) or miR-28-5p and PGL3-*NM23-H1*-WT. Experiment was also performed with a construct in which the binding site was mutated. NS, not statistically significant (Student *t* test).



Supplementary Figure 9. Luciferase activity of HCT116 cells cotransfected with SCR (n = 1) or miR-28-5p and PGL3-*CCND1*-WT. Experiment was also performed with a construct in which the binding site was mutated (***$P < .005$, Student *t* test).



Supplementary Figure 11. In situ hybridization analysis for miR-28-5p and miR-28-3p in normal colon tissue. Frozen tissue sections were digested with proteinase K and loaded onto Ventan Discovery Ultra. The tissue slides were incubated with double-DIG labeled miRCURY LNA Detection probe, and the digoxigenin was detected with a polyclonal anti-DIG antibody and UltraMap Blue anti-Ms Detection Kit. H&E staining was performed. Microscopy images were obtained with a magnification of 100×.

Supplementary Table 1. Efficiency of Taqman Assay for miR-28-5p (Assay Number 000411), miR-28-3p (Assay Number 002446), and snRNA U6 (Assay Number 001973) Using the C_t Slope Method

Taqman assay	R^2	Slope	Efficiency[a]
miR-28-5p	0.9979	−3.5601	0.91
miR-28-3p	0.9997	−3.2352	1.04
snRNA U6	0.9956	−3.2498	1.03

[a]PCR efficiency was determined using the formula: Efficiency = $10^{-1/\text{slope}} - 1$.

Supplementary Table 2. miRNA-28-5p and miR-25-3p Expression (Using ΔC_t Method) in Normal Colon and Colorectal Cancer Samples for 2 Independent Sets

	Mean	SEM
miR-28-5p		
First set of samples		
Normal	0.058	0.003
Tumor	0.044	0.003
MSS	0.043	0.004
MSI	0.046	0.004
Second set of samples		
Normal	0.238	0.025
Tumor	0.151	0.019
miR-28-3p		
First set of samples		
Normal	0.029	0.001
Tumor	0.022	0.002
MSS	0.022	0.002
MSI	0.022	0.002
Second set of samples		
Normal	0.319	0.028
Tumor	0.161	0.017

NOTE. Values were normalized to small nuclear RNA U6.
SEM, standard error of mean.

Supplementary Table 3. miR-28-5p Expression in Colorectal Cancer Compared With Normal Colon in 2 Independent Sets of Samples[a]

Gene	Type	Reaction efficiency	Samples	Expression	Standard error	95% CI	*P* value	Result
miR-28-5p	Target	0.9094	First set (paired)	0.620	0.389−0.971	0.258−2.380	.000	Down
			Second set (paired)	0.641	0.320−1.254	0.173−2.544	.003	Down
			First set (all)	0.711	0.441−1.209	0.211−2.282	.001	Down
U8	Reference	1.0309		1				

NOTE. Small nuclear RNA U6 was used as a reference gene.
CI, confidence interval.
[a]Using Pfaffl Method, REST 2009 Software (Qiagen, V2.0.13).

Supplementary Table 4. Sequences of Mature Human miR-28-5p and miR-28-3p According to miRBase, Primers Used to Amplify miR-28, and Primers Used to Generate PGL3 Constructs for Luciferase Assays and to Generate Deletions in the miRNA-Binding Site

	Sequences
Mature miRNA	
hsa-miR-28-5p	AAGGAGCUCACAGUCUAUUGAG
hsa-miR-28-3p	CACUAGAUUGUGAGCUCCUGGA
Primers	
mir-28-Fw-BamHI	CGGATCCAGGCCCTTCAAGGACTTTCT
miR-28-Rv-EcoRI	CGAATTCACAGAGCTCCTGCTGTGTCA
Primer for PGL3 construct	
CCND1_XbaI_Fw	CGTCTAGAGTCCCACTCCTACGATACGC
CCND1_XbaI_Rv	CGTCTAGACTTGCCTCAAAGTCCTGCTT
HOXB3_XbaI_Fw	CGTCTAGAAAGGACATTGTGTTTCCTGTCA
HOXB3_XbaI_Rv	CGTCTAGACAAAGAAAGTTCCAAGAGGGAAT
NM23_XbaI_Fw	CGTCTAGAGCAGACCACATTGCTTTTCA
NM23_XbaI_Rv	CGTCTAGAAACCAACTCAATGAATCCTATGC
Primers for mutagenesis	
CCND1_Mutagenesis_Fw	GGTTCAACCCACAGCTACTTGCATATTCTAAAACCATTCCAT
CCND1_Mutagenesis_Rv	ATGGAATGGTTTTAGAATATGCAAGTAGCTGTGGGTTGAACC
HOXB3_Mutagenesis_Fw	GTTCTAAAAGGCATGAACTCATCGTCACTGTATAGTCCTG
HOXB3_Mutagenesis_Rv	CAGGACTATACAGTGACGATGAGTTCATGCCTTTTAGAAC
NM23_Mutagenesis_Fw	AGAGGACCAGGCTGTAGGATATTTACAGGAACTTCATC
NM23_Mutagenesis_Rv	GATGAAGTTCCTGTAAATATCCTACAGCCTGGTCCTCT

NOTE. Restriction sites for endonucleases are underlined.

3. Discussion

3. General Discussion

Due to the role of miRNAs in controlling expression of protein-coding mRNAs or non-protein-coding transcripts, any deregulation in miRNA levels affects a high number of targets, which might lead to consequences in an extensive number of cellular functions. Herein, I highlight the relevance of miRNAs in colorectal cancer pathogenesis. In addition, I discuss how the innovative data concerning the differential role of the strand-specific mature miRNAs originated from the same pre-miRNA (specifically, the pre-miR-28 that originates miR-28-5p and miR-28-3p) holds a tremendous impact on miRNA-based therapy design.

3.1 The Broad Relevance of Deregulated microRNAs in Colorectal Cancer

In 2004, Lui and Calin *et al.* were the first to describe a microarray-based genome-wide miRNA profiling [1, 2]. The study analyzed RNA from 20 normal human tissues (colorectal tissue was not included) and revealed tissue-specific miRNA expression signatures [1, 2]. In 2005, Lu *et al.* performed the first bead-based genome-wide miRNA profiling in a large panel of samples that included varied human tissues (including colorectal) [2, 3]. When analyzing the miRNA expression profiles, the authors were able to classify human tumors based on the developmental lineage and the differentiation state [2, 3]. This study also concluded that there was a general miRNA downregulation in tumors versus normal tissues [3]. Few months later, Volinia *et al.* performed a large-scale miRNA profiling analysis using samples from 6 different tumor types and established tumor-specific signatures, which further proved the involvement of miRNAs in cancer pathogenesis. However, the authors did not found the general miRNA downregulation described in Lu *et al.* study [4]. With the miRNA field getting more attention from the researchers, genome-wide miRNA profiles became common. Commercial miRNA arrays were generalized and nowadays include a larger number of miRNAs. Regarding colorectal cancer, miRNA wide-range profiling analysis are not only used to distinguish miRNA levels between normal and tumor

tissue but also to obtain profiles that are able to distinguish tumors according to the clinico-pathologic or molecular features [5]. Importantly, different tumor types have different miRNA signatures.

Some protein coding genes are known to display antagonistic functions in oncogenesis depending on the cellular context. This is the case of Maf b-Zip transcription factors [6], p21Cip1/Waf1 [7] and WT1 [8], among others. The same dual role can occur for miRNAs. Therefore, depending on the tissue and cellular type, a miRNA can function as an oncogene or as a tumor suppressor. Depending on the tumor type, miRNA targets might be different, which can partially explain this duality. Our work was the first to show that miR-28 (-5p and -3p) expression was downregulated in colorectal cancer compared with normal colon tissue. Our conclusion is supported by the results obtained in two independent sets of samples. Moreover, a recent profiling study in colorectal cancer using miRNA microarray data further strengthened our results by showing miR-28 (-5p and -3p) downregulation in colorectal cancer [9]. Before our study, the analysis of miR-28 expression had only been described for other tumor or cell types and found miR-28 to be upregulated in tumors compared with normal tissues/cells [10-12]. Additionally, these reports did not differentiate between the two miR-28 mature forms. In the literature, other miRNAs are described to have a dual oncogenic/tumor suppressor function, depending on the tissue or cellular type: the same miRNA can be upregulated in some tumor types but downregulated in others. This is the case of miR-15a/miR-16-1 cluster that is downregulated in CLL, diffuse large B-cell lymphoma, multiple myeloma, pituitary adenoma, prostate and pancreatic cancer while upregulated in nasopharyngeal carcinoma [13]. miR-15a/miR-16-1 cluster has distinct targets in the different tumor types [13] and it is particularly well know that this cluster is involved in apoptosis in leukemia by directly targeting the anti-apoptotic Bcl-2 mRNA (miR-15a/miR-16-1 cluster as a tumor suppressor) [14]; whereas upregulated miR-15a/miR-16-1 cluster in nasopharyngeal carcinoma has the tumor suppressor BRCA1 as a target (miR-15a/miR-16-1 cluster as an “oncomiR”). In the same line of evidence, miR-17-92 cluster is frequently overexpressed in lung, colon, small-cell lung cancer, lymphoma, multiple myeloma and medulloblastoma [13, 14], while LOH in miR-17-92 locus is present in

patients with melanoma, ovarian and breast cancer [13]. The same dualism occurs in different tumor types regarding two miRNA families: let-7 and miR-29 family [13]. Also, miR-125a/b is downregulated in glioblastoma, breast, prostate and ovarian cancer while upregulated in myelodysplastic syndrome, in acute myeloid leukemia with t(2;11)(p21;q23) and in urothelial carcinoma [13]. In conclusion, based on the literature it is not uncommon to identify miRNAs´ dual role as tumor suppressor or as oncogene, according to the cellular context. Consequently, the targets and functions that miRNAs play in cancer are always dependent on the tumor type we are analyzing.

In our work, described in Chapter 2, we found that miR-28-overexpressing colorectal cancer cells decreased tumor growth in mice xenografts. Other miRNAs that suppress/decrease colorectal tumorigenicity in *in vivo* models include, miR-148b [16], let-7a [17], let-7c [18], miR-143 [19, 20], miR-145 [20], miR-26b [21], miR-133b [22], miR-491 [23], while miR-211 [24] and miR-95 [25] potentiates tumor growth *in vivo*. Presently, the *in vivo* effects that miR-28 causes in other tumor types are not yet described in the literature. The tumor suppressor behavior we found for miR-28 can be partially explained by the miR-28-5p effects on decreasing cell proliferation, induction of apoptosis and G1-arrest when colorectal cancer cells are transfected with miR-28-5p. Furthermore, we found Cyclin D1 as a miR-28-5p target in HCT116 colorectal cancer cell line. The protein encoded by CCND1 (Cyclin D1) forms a complex with and functions as a regulatory subunit of CDK4 or CDK6, whose activity is required for cell cycle G1/S transition. The study described in Chapter 2 was the first to report an inverse relation between miR-28-5p expression levels and Cyclin D1 protein levels in cancer cells. Regarding colorectal cancer solely, an analysis of the present literature can provide us a larger spectrum on how miRNAs regulate G1 cell cycle transition in colorectal cancer. Nie *et al.* found Cyclin D1 was a target of miR-365, which is a miRNA downregulated in colorectal cancer that is able to inhibit cell cycle progression upon restoration of it expression [26]. miR-145 directly regulates CDK6, a major Cyclin D1-dependent kinase [20]. Additionally, this miRNA controls the expression of other G1 cell-cycle regulators as CCND2 and E2F3 [20]. Faltejskova *et al.* found that overexpression of miR-378, miR-422a and miR-375 inhibited G1/S transition but no targets were described [27]. Another study showed that miR-342 overexpression led to

G(0)/G(1) cell cycle arrest and that it was associated with reduced levels of Cyclin E and CDK2 protein in colorectal cancer cells. On the other hand, after miR-342 transfection, Cyclin D1 and CDK6 protein expression levels remained unchanged [28]. Ectopic expression of miR-185 induces G1-arrest by directly interfering with RhoA and Cdc42 levels. Both RhoA and CDC42 proteins are well known cell cycle regulators that can control Cyclin D1, among other proteins [29]. Additionally, CDC42 has also been described as regulated by miR-137 [30] and by miR-21 in colorectal cancer cells [31]. Several studies associate miRNAs in colorectal cancer to cell cycle regulation: Luo *et al.* [32] reported that inhibition of miR-17 induced G0/G1 arrest and targets RND3; Wang *et al.* [33] proved that let-7a enhanced expression reduced G0/G1 arrest and that it targets NIRF; and Baraniski *et al.* [34] showed that miR-30a-5p causes G-1 arrest by targeting DTL. However, any of these studies reported direct targets of the cyclin-dependent kinase (CDK)-cyclin complexes [32, 33, 34].

Additionally, we found that HOXB3 was other target of miR-28-5p. Homeobox genes of the Hox family were originally discovered in controlling body structure and development of *Drosophila melanogaster* [35]. Hox genes are located contiguously in clusters and encode transcription factors [35]. In humans, 39 Hox genes were identified [35]. Hox genes are not only expressed during embryogenesis, but also throughout postnatal life and aberrant Hox gene expression is found in cancer [35, 36]. It is well know that Hox genes can be regulated by epigenetic mechanisms as well as by miRNAs [35]. miR-10a targets HOXA3, HOXD10 [37] and HOXA1, according to luciferase experimental results [38]. miR-196 represses HOXB8 through mRNA cleavage [39]. Both miRNAs, miR-10a and miR-196b, are located within the Hox gene clusters locus [35]. Hox gene activity is tissue specific [35]. In colorectal cancer, HoxB3 is upregulated in left-sided large intestine carcinomas compared to normal tissue [40]. The function of HoxB3 in colorectal cancer was not described so far. However, taking into account the role of Hox genes in other tumor types, we can speculate it might play a role in proliferation and apoptosis [35]. For example, Morgan *et al.* designed a novel cell-permeable peptide, named HXR9, to interfere with the capacity of Hox to bind to PBX co-factor, and demonstrated that HXR9 caused apoptosis in melanoma cells *in vitro* and tumor growth retardation in a mouse melanoma cell line *in vivo* [41]. Later,

Plowright *et al.* used the same peptide to study the role of Hox in NSCLC where several Hox genes were upregulated compared with normal adjacent tissue, including HoxB3, and proved that blocking Hox activity triggers apoptosis in NSCLC cells *in vitro* and blocks tumor growth of a NSCLC cell line *in vivo* [42]. The same result was obtained in an ovarian cell line [43]. Furthermore, Costa *et al.* showed that HOXA9 (one of the genes of Hox family) was activated in glioblastoma versus normal brain tissue and that it increases cell proliferation and inhibits apoptosis [44]. Together, studies support a role of Hox genes in regulating apoptosis and inducing proliferation.

In the study described in Chapter 2, we also found miR-28 as being a metastasis promoter in colorectal cancer cells *in vivo*. Several miRNAs have been directly implicated in the metastatic process of colorectal cancer, being pro-metastatic or anti-metastatic [45]. EMT is a signature of the metastatic process [46]. From all miRNAs involved in EMT, miR-200 family is probably the most recognized as a master regulator of epithelial phenotype [47]. Members of miR-200 family target ZEB1 and ZEB2/SIP1 transcription factors, which in turn strongly repress E-cadherin transcription. E-cadherin loss is a characteristic of EMT [46, 48]. Inhibition of the miR-200 family members was sufficient to upregulate ZEB1 and/or ZEB2, to reduce E-cadherin and to induce EMT in cancer cell lines [46, 48, 49] while upregulation of those miRNAs triggered the reverse process [46, 48, 49]. Remarkably, several members of miRNA-200 family were downregulated in cells that had undergone EMT in response to TGF-β [47]. Notably, in a feedback loop mechanism, ZEB1 repressed two of the miR-200 members, namely miR-200c and miR-141 [49, 50]. Interestingly, in colorectal carcinomas, ZEB1 is aberrantly up-regulated in dedifferentiated tumor cells at the invasive front, which associates with loss of the basement membrane [51]. In addition, SIX1 - a protein that induces EMT in colorectal cancer cells - is able to inhibit the transcriptional activity of the miR-200 family in a colorectal cell line [52]. Also, in the colorectal cancer cell lines SW480 and SW620, it was proved the inhibition of ZEB1 by miR-200c [53]. Although several of these studies were performed in colorectal cancer cell lines, literature reports comparing miR-200c levels between normal and colorectal cancer specimens showed an upregulation of miR-200c in the colorectal tissue [54], which is contradictory with the potential anti-metastatic role. However, miR-200 levels

should be determined analyzing only in the dedifferentiated tumor cells at the invasive front of the tumor.

The process that initiates with the EMT results in enhanced cell migration and invasion [55]. The migratory and invasive phenotype is a feature that characterizes metastasis and this topic has been explored by researchers. miR-141 is considered an anti-metastatic gene in colorectal cancer cells *in vitro* because it inhibits migration and invasion [56]. Also, miR-345 is considered an anti-metastatic gene. miR-345 promoter methylation led to it downregulation in colorectal cancer and low miR-345 expression levels are associated with lymph node metastasis. When miRNA levels were restored, cell invasiveness through membrane of transwell chamber significantly decreased [57]. Likewise, miR-137 [58] and let-7c [59] blocked invasion of the colorectal cancer cells. Wang *et al.* reported miR-31 suppression decreases migration but increases invasion in a colon cell line; therefore, the effect of miR-31 in metastasis remains inconclusive [60].

When exploring the functional role of miRNAs in the metastatic process, literature studies that used both *in vitro* and *in vivo* approaches are much less common than studies that used only *in vitro* methodologies, probably to the higher cost of the *in vivo* models. Although the mice model does not reproduce exactly the human biology, it can provide us further information on the miRNAs biological effects. In fact, the combination of *in vitro* and *in vivo* methods is, in our opinion, a good strategy in order to answer to the biologic questions that are proposed in this thesis. Regarding the study of miRNA role in metastasis of colorectal cancer cells, only Schimanski *et al.* [61], Wang *et al.* [62] and Lui *et al.* [63] gave a step forward by using mice models besides the *in vitro* approaches. Schimanski *et al.* demonstrated that miR-196a expression increased migration and invasion in colon cancer cells and it increased lung metastasis in mice xenografts. By doing so, the authors performed the first functional study on colorectal metastasis *in vivo*. Importantly, miR-196a did not have an impact neither in proliferation nor in apoptosis, reinforcing miR-196a as an pro-metastatic gene [61]. Also Wang *et al.* showed that lung metastasis incidence in nude mice was lower in a colon cell line stably expressing miR-342 vector than in mice injected with the empty control vector [62]. Lui *et al.* demonstrated that a colorectal cell line

overexpressing miR-499-5p promoted lung and liver metastasis, probably thought the targeting of FOXO4 and PDCD4 [63]. Several *in vitro* studies have also demonstrated the implication of miRNAs in colorectal cancer migration/invasion as it is the case of Cottonham *et al.* study that demonstrated miR-21 and miR-31 involvement in cell motility and invasion by direct suppressing TIAM1 in colon carcinoma cells [64]. Finally, several other miRNAs such as miR-185 and miR-133b have also been proposed as implicated in the metastatic process [65] but were not functionally validated yet.

The role of the miRNAs on EMT, migration, invasion and metastatic tumor formation is only possible due to the miRNA function of targeting mRNAs, which are directly or indirectly linked with the molecular pathways that regulate metastatic process. In the work described in this thesis, we found NM23-H1 to be targeted by miR-28-3p. The anti-metastatic properties of NM23 are described since 1988 and it is considered the first metastatic suppressor gene to be described in the literature [66]. Until present, our study is the first describing NM23 regulation by miRNAs.

3.2 Therapeutic Implications of microRNAs: Relevance of microRNA Strand-Specific Mechanisms

miRNAs are deregulated in human cancers and are crucial regulators of cancer development and progression, which turns miRNAs into attractive therapeutic targets [67, 68]. Due to the fact that a single miRNA might target several mRNAs, we would be able to regulate the expression of several genes in core signaling pathways involved in cancer by interfering with the expression of a single miRNA [67, 68], and this is particularly attractive in the field of oncology [69]. For example, miR-21 targets multiple important components of the p53, TGF-β, and mitochondrial apoptosis tumor-suppressive pathways [69, 70] and therefore constitutes an important therapeutic target. However, the fact that a miRNA can have multiple targets can also constitute a disadvantage due to the potential side-effects. Thus, any potential miRNA target needs not only to be predicted by computer algorithms but also to be experimentally validated [67]. Off-target effects and lack of efficient delivery systems are some of the challenges facing RNA-based therapies [67, 68]. It is necessary to

overcome these issues so that small RNAs turn from the successful research tools, for gene functional studies that we currently use, into clinical therapeutic tools [67]. Strategies to regulate both oncogenic and tumor suppressor miRNAs are being developed.

As progress has been made toward the design and delivery of short interfering and short hairpin RNAs for therapeutic gene silencing, downregulation of a single oncogenic miRNA became an attractive strategy. For this propose, methodologies to inhibit miRNA function are being developed and these include the use of anti-miRNA oligonucleotides (AMOs)/antagomiRs (such as 2-O-methyl AMOs, 2-O-methoxyethyl AMOs, LNAs and other modified oligonucleotides), miRNA sponges, and miRNA masking [67, 71] (Figure 7). Anti-miRNA oligonucleotides are synthetic antisense nucleotides complementary to miRNAs that bind to it and inhibit the interaction between miRNA and their mRNA targets [72]. Lanford *et al.* used LNAs complementary to miR-122 to treat chimpanzees infected with chronic hepatitis C virus infection [73]. This treatment is now is phase II clinical trials [74]. miRNA sponges [72] are artificial competitive inhibitors that function in a similar way as the natural decoy ceRNAs. These are vectors encoding transcripts containing multiple, tandem binding sites to a miRNA of interest, expressed under strong promoters. miRNA sponges will compete with the miRNA target and derepress the miRNA-mRNA binding, by sequestering the miRNAs. Remarkably, a single sponge can be used to block multiple miRNAs, those who will bind to the seed region encoded by the sponge [72, 75, 76]. A dynamic technology developed in *Drosophila* based on miRNA sponges, named miR-SP, allows *in vivo* miRNA silencing, with precise *in vivo* spatial resolution [72, 77]. Finally, the miRNA masking approach is based in single-stranded 2'-O-methyl-modified oligonucleotide (or other chemically modified oligonucleotide) fully complementary to the miRNA binding site. miRNA mask does not bind directly to the miRNA but instead binds to the mRNA target of a specific miRNA blocking the access of the miRNA to its´ target. This method is gene-specific and miRNA-specific [72, 78]. Choi *et al.* tested this approach *in vivo* in zebra fish [72, 78].

Although studies on oncogenic miRNAs are extremely important and despite the contribution of oncogenic miRNAs to tumorigenesis (that is undisputable), some

studies showed that there is a general repression of mature miRNAs in human cancers [3, 13]. The global miRNA loss enhances tumorigenesis, as demonstrated by Kumar *et al.* in a study where cancer cells expressing shRNAs targeting three different components of the miRNA processing machinery were used to induce a decrease in miRNA maturation [80]. Also, Dicer knockout enhanced tumor development in mice [80]. Therefore, therapies that are able to contradict miRNA downregulation and to restore miRNA levels are most needed [81]. miRNA levels can be restored ("miRNA replacement therapy") using two approaches: 1) direct delivery of miRNA oligonucleotide mimics - synthetic RNA duplexes consisting of a guide strand that is identical to the mature miRNA sequence and a passenger strand partially or fully complementary to the guide strand, which are generally chemically modified to reduce oligonucleotide degradation and enhance it stability, permeability and activity; 2) introduction of the miRNA gene via an expression vector (e.g., plasmid, virus) [67, 69] (Figure 7). Small molecules that can reverse the epigenetic changes caused on miRNAs can also be used [69]. The research study described in this thesis embraces this issue and provides insight for the design of strategies to restore tumor suppressor miRNA levels. Henry *et al.* study compared miRNA mimics oligonucleotides and expression vectors for miRNA mimics [69]. The authors concluded that miRNA mimics oligonucleotide are commonly used for *in vitro* transfections because of the simplicity of the transfection (using lipid-based reagents or electroporation) [70]. Currently, miRNA oligonucleotide mimics are also becoming more widely used *in vivo*. However, the cost to synthetize and purify these oligonucleotides is high, and their expression after transfection is not long lasting, which constitutes the main disadvantage [69]. On the other hand, when miRNAs are delivered into the cells through a vector and miRNAs are introduced into the nucleus, the miRNA expression after transfection lasts much longer than when using miRNA oligonucleotide mimics [69]. The authors also state that, when using this strategy, the likelihood of off-targets effect is reduced because the miRNA is processed normally through the cell machinery and the -5p and -3p strands are completely natural to the cell [69]. The novelty of the work described in this thesis complements exactly this issue. If the miRNA introduced into the cell is processed by the cell machinery and originates only one single mature RNA, or two mature RNAs (-5p and -3p strands) with cooperative functions (e.g., both strands

decrease proliferation) the use of vector delivery strategy is advantageous. But if the miRNA introduced into the cell originates two mature RNAs (-5p and -3p strands) with distinct functions, this will create undesired side effects. Our work elucidates that miR-28 delivery decreases tumor growth but has the undesired effect of increasing metastasis, and that this is probably caused by the distinct -5p and -3p effects on colorectal cells.

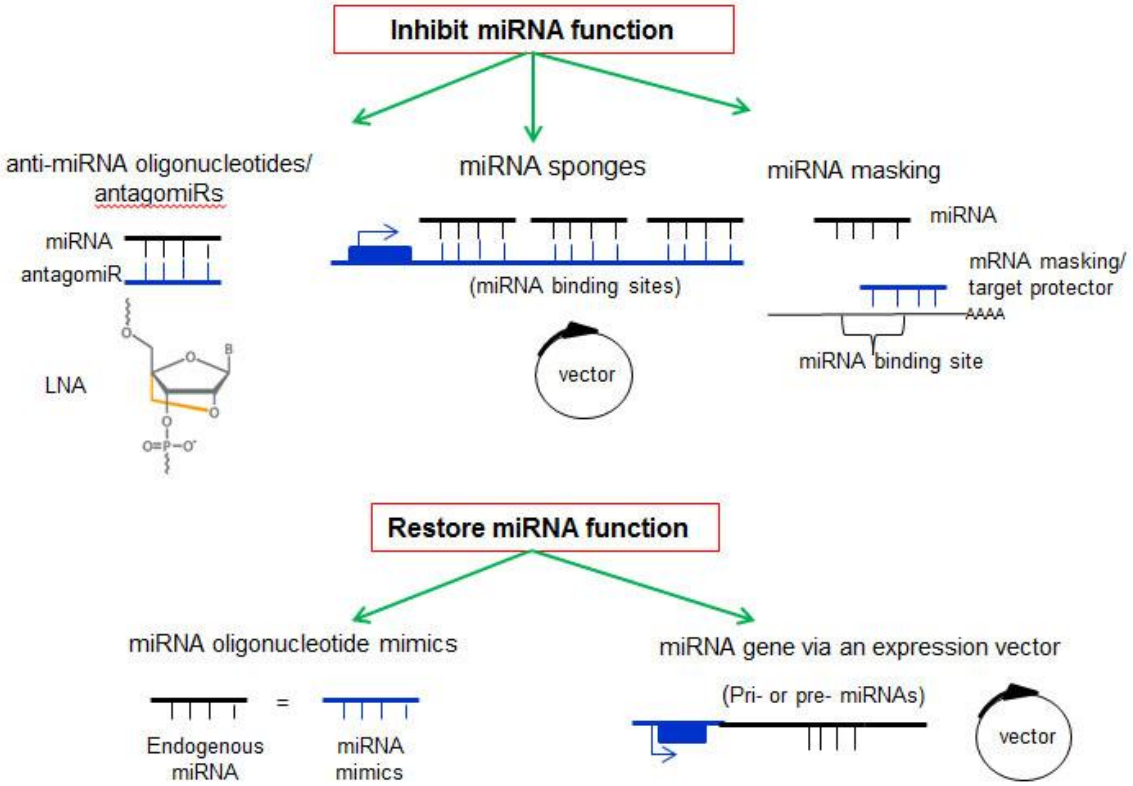


Figure 7. Schematic representation of strategies to either decrease or increase miRNA levels. (LNA: locked nucleic acid - adapted from http://www.exiqon.com/lna-technology)

Numerous studies used the above described strategies for *in vivo* miRNA delivery into cancer cells. Regarding miRNA oligonucleotide mimics *in vivo* delivery and focusing on colorectal cancer in particular, Kitade *et al.* tested the capacity of miR-143, which is downregulated in colorectal cancer compared with normal tissue, as a therapeutic target [82]. The authors chemically modified miR-143 oligonucleotide mimics by introducing an aromatic benzene-pyridine compound to the 3'-overhang

region of the RNA-strand, which consequently improved its nuclease resistance compared with the commercially available miR-143 [82]. Then, the authors tested it clinical application and found xenografted tumors of colorectal cancer were decreased after weekly intravenous injections (5 weeks treatment) compared with the control animals [82]. Although this is a single example on the use of miRNA mimics oligonucleotide in colorectal cancer *in vivo*, numerous studies have been published in other tumor types, suggesting the importance for cancer treatment [69]. Regarding miRNA vector *in vivo* delivery, no studies on colorectal cancer were reported so far, but only in other tumor types [69]. The first study demonstrating that therapeutic delivery of a tumor suppressor miRNA through a vector could be effective *in vivo* was described in 2009 [71]. Kota *et al.* used a mouse model of hepatocarcinoma, in which MYC expression was induced by tetracycline, to demonstrate that therapeutic delivery of miR-26a through an adeno-associated virus vector suppresses tumorigenesis. Importantly, the adeno-associated virus system used was effective and no measurable liver toxicity was found after systemic administration [71, 83]. In lung cancer, Trang *et al.* provided proof-of-concept for *let-7* replacement therapy [84]. The authors analyzed the impact of intranasal administration of a lentiviral vector expressing the tumor suppressor let-7a and, in established KRAS dependent mice lung tumors, observed tumor remission in the lentivirus-treated group of mice compared with the baseline and lentivirus-control groups [84]. Another example of the modulation of miRNA activity as a therapeutic strategy *in vivo* is Wang *et al.* study [85]. The authors constructed an adenovirus expressing miR-101 that was intratumoraly injected into a gastric xenograft tumor model [85]. The tumors in the mice group treated with miR-101-adenovirus were significantly smaller than tumors treated with the control-adenovirus, and any mice showed notable toxic effect [85]. In summary, miRNA replacement therapy holds potential as a promising approach in cancer treatment.

3.3 Concluding remarks

Each of the miRNAs deregulated in cancer holds great potential to act as a therapeutic target. This is the case of miR-28 that we found deregulated in colorectal tumors. miR-28 connects with the widespread network of miRNAs that works together to influence

tumors´ proliferation, apoptosis, cell cycle, or metastasis, by targeting mRNAs implicated in several pathways. Therefore, modulation of miRNA levels constitutes a powerful tool to control cellular gene expression and cellular biology. Although much still needs to be done to translate miRNA-based therapies into the clinical practice, several therapeutic strategies are being developed. For cases in which stem-loop miRNAs encode two mature miRNAs, as it is the case of pre-miR-28, studies analyzing the function of -5p and -3p strands need to be perform. This, together with the analysis of the overall consequences of the pre-miRNA, is crucial for the design of miRNA-based therapies, due to the possibility of undesirable side-effects.

Taken together, the study presented here encloses the following major conclusions:

- miR-28 is downregulated in human colorectal cancer compared with normal colorectal tissue.
- miR-28-5p decreases proliferation, increases apoptosis, causes G1-arrest, and decreases migration and invasion in colorectal cancer cells.
- miR-28-3p has no effect on proliferation but increases migration and invasion in colorectal cancer cells.
- Overall, pre-miR-28 decreases tumor growth but increases metastasis *in vivo*.
- We experimentally proved that miR-28-5p represses Cyclin D1 and HOXB3 expression, while miR-28-3p targets NM23-H1 in colorectal cancer cells. Computational algorithm predictions indicate that hundreds of mRNAs can be regulated exclusively by miR-28-5p, exclusively by miR-28-3p or by both.
- Hairpin loop pre-miR-28 encodes miR-28-5p and miR-28-3p mature miRNAs, which have distinct functions. All other pre-miRNAs in which both -5p and -3p strands are active should be functionally analysed due to possible inconvenient effects. The possible dual role of -5p and -3p strands has direct implications on the design of miRNA-based therapeutic approaches.

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Appendix

The results presented throughout this chapter were:

(i) Published as original articles, review articles or commentaries in international peer reviewed journals:

PAPER I - Almeida MI, Reis RM, Calin GA. MicroRNAs and metastases—the neuroblastoma link. Cancer biology & therapy. 2010;9(6):453-4.

PAPER II - Almeida MI, Reis RM, Calin GA. MYC-microRNA-9-metastasis connection in breast cancer. Cell Research. 2010;20(6):603-4.

PAPER III - Almeida MI, Reis RM, Calin GA. BRCA1, microRNAs and cancer predisposition: challenging the dogma. Cell Cycle. 2011;10(3):377.

PAPER IV - Zheng H, Song F, Zhang L, Yang D, Ji P, Wang Y, Almeida M, Calin GA, Hao X, Wei Q, Zhang W, Chen K. Genetic variants at the miR-124 binding site on the cytoskeleton-organizing IQGAP1 gene confer differential predisposition to breast cancer. International Journal Oncology. 2011;38(4):1153-61.

PAPER V - Almeida MI, Reis RM, Calin GA. MicroRNA history: discovery, recent applications, and next frontiers. Mutation Research. 2011;717(1-2):1-8.

PAPER VI - Olaru AV, Ghiaur G, Yamanaka S, Luvsanjav D, An F, Popescu I, Alexandrescu S, Allen S, Pawlik TM, Torbenson M, Georgiades C, Roberts LR, Gores GJ, Ferguson-Smith A, Almeida MI, Calin GA, Mezey E, Selaru FM. MicroRNA down-regulated in human cholangiocarcinoma control cell cycle through multiple targets involved in the G1/S checkpoint. Hepatology. 2011;54(6):2089-98.

PAPER VII - Spizzo R, Almeida MI, Colombatti A, Calin GA. Long non-coding RNAs and cancer: a new frontier of translational research? Oncogene. 2012. (*in press*)

PAPER VIII- Almeida MI, Reis RM, Calin GA. Decoy activity through microRNAs: the therapeutic implications. Expert Opinion on Biological Therapy. 2012. (*in press*)

PAPER IX - Le Xiao-Feng, Almeida MI, Mao W, Spizzo R, Rossi S, Nicoloso MS, Zhang S, Wu Y, Calin GA, Bast Jr RC. Modulation of MicroRNA-194 and Cell Migration by HER2-Targeting Trastuzumab in Breast Cancer. PLoS One. 2012. (*in press*)

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MicroRNAs and metastases

The neuroblastoma link

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MicroRNAs (miRNAs) are small noncoding RNAs of approximately 22 nucleotides in length that regulate gene expression post-transcriptionally. These small RNAs are fundamental regulators of several cellular processes, such as differentiation, development, apoptosis, proliferation, cell cycle regulation and metabolism, through the binding to 3' untranslated regions, coding sequence or 5' untranslated regions of target messenger RNAs (mRNAs), preventing their translation or causing their degradation.[1] A modest change in only one miRNA will affect multiple mRNA targets; consequently, the deregulation of miRNAs has important consequences to the cellular homeostatic stability, and aberrant miRNAs expression patterns have been described in several types of cancer.[2] Recently, miRNAs have been implicated in the metastatic process of several tumors such as human breast and colorectal cancers[3] and, as reported this issue of *Cancer Biology & Therapy* by Guo et al. in neuroblastoma.[4] These are extracranial solid tumors, arising from neural crest cells, that are most common in infants and children; metastasis, the main cause of death, is present at the time of diagnosis in approximately 60% of patients.[5] Metastatic disease may cause bone pain, bone marrow suppression and weight loss, but some patients do not present symptoms.[6] Therefore, the study by Guo et al. is important for the identification of specific miRNAs responsible for metastasis not only to better understand the molecular behavior of theses tumors but also to identify markers of early diagnosis/prognosis, and in the future, possible new therapeutic targets.

Using a heterotopic transplant mouse model of neuroblastoma and a microRNA microarray analysis approach, Guo et al. identified 54 miRNAs differentially expressed between primary and metastatic neuroblastoma tumors.[4] Three of the top ten downregulated miRNAs in this study, namely miR-7, miR-338-3p and the let-7 family, have an anti-metastatic role in other tumors types.[7] Specifically, in breast cancer, Reddy et al. described that endogenous miR-7 expression is positively regulated by the homeodomain transcription factor HoxD10, a gene that has been linked with the invasive and metastatic potential in human breast cancer cells. In this breast cancer cell line, the authors showed that miR-7 introduction suppresses motility, invasiveness, anchorage independence and tumorigenesis.[8] Regarding miR-338-3p, it has been suggested that this microRNA contributes to the formation of basolateral polarity in epithelial cells, which can be important for metastasis prevention.[9] Concerning the let-7 family, several studies demonstrate that it can inhibit tumorigenesis and metastasis as it targets important oncogenes such as RAS, MYC and HMGA2. Interestingly, MYC and let-7 are involved in a feedback loop since MYC can be a target of let-7 and at the same time can control its expression. In breast tumor cells, Dangi-Garimella et al. demonstrate that RKIP represses metastasis, in part, through a signalling cascade that involves the inhibition of MAPK, MYC and LIN28, leading

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to the induction of let-7 and the consequent downregulation of its targets.[10]

In the present reported study, Guo et al. also identified several miRNAs whose association with metastases was not previously described. In futures studies, it will be important to further analyse those miRNAs with the purpose of understanding their functional role in metastisation. In addition to the identification of miRNAs involved in the neuroblastoma metastatic process, and using computer-aided algorithms, Guo et al. suggest potential mRNA targets that can be regulated by those miRNAs. Interestingly, some of those targets have been described as deregulated in neuroblastoma.[4] For example, according to Guo et al. miR-29-a/b is overexpressed in metastatic neuroblastoma and one of the predicted targets of this microRNA is CASP8. The absence of this apoptosis-mediated protein has been implicated in neuroblastoma metastasis in vivo. In chick embryos, Stupack et al. detected lung and bone marrow neuroblastoma metastases mostly in embryos bearing tumors deficient in CASP8 when compared to CAPS8-positive tumors. Furthermore, reconstitution of CASP8 expression significantly suppressed metastasis.[11] The authors propose that unligated or antagonised integrins on the neuroblastoma cell surface activate a CASP8-dependent checkpoint and block cell invasion into an inappropriate microenvironment.[11] This mechanism is inactive in the absence of CASP8.[11,12] Interestingly, integrins are also predicted to be a target of miR-29a/b, which is overexpressed in metastatic versus primary neuroblastoma.[4] In addition to CASP8 deletion and methylation,[12] it is tempting to speculate that another mechanism by which CASP8 is downregulated in metastatic neuroblastoma is through miR-29a/b overexpression. However, it is imperative to validate this hypothesis through functional studies.

It will be important in future studies to functionally validate the interference between the other miRNAs that were differently expressed (primary versus metastatic NB) in the Guo et al. study and the predicted targets. In humans, neuroblastoma can metastasize to bone, bone marrow, lung, liver and/or non-contigous lymph nodes.[13] It will be also interesting to analyse differences at the microRNA level between the different metastatic sites.

In conclusion, the identification of differentially expressed microRNA in the metastasis of neuroblastoma is an important step towards the understanding of the metastatic process in this disease.

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RESEARCH HIGHLIGHT

MYC-microRNA-9-metastasis connection in breast cancer

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Metastasis accounts for more than 90% of cancer patients' mortality. The metastatic process involves multiple steps [1]. Initially, cancer cells from the primary tumor invade adjacent stroma. To acquire this capacity, cells undergo a process called epithelial-mesenchymal transition (EMT), in which cells in response to signals from the surrounding stroma, undergo a switch between cell phenotypes and acquire mesenchymal properties and show reduced intercellular adhesion, allowing cells to become motile. Then cells enter systemic circulation, either through the blood or lymph, and finally extravasate into the parenchyma of distant tissues, where they form micrometastasis and proliferate to form secondary tumors [2].

MicroRNAs (miRNAs) are a class of non-coding RNAs with approximately 22 nt length that regulate gene expression post-transcriptionally by binding to 3′ untranslated region (UTR), coding sequence or 5′ UTR of target messenger RNAs (mRNAs), leading to inhibition of translation or mRNA degradation [3]. Tumors present aberrant expression of miRNAs patterns. miRNAs have been described as regulators of several biological processes such as apoptosis, proliferation, differentiation, and more recently, metastasis [4]. miRNAs can function either as suppressors or promoters of metastasis according to their mRNA targets [1, 2]. Elucidation of the role of miRNAs in metastasis can lead to a better understanding of this process and consequently lead to new treatments for patients with late-stage tumors.

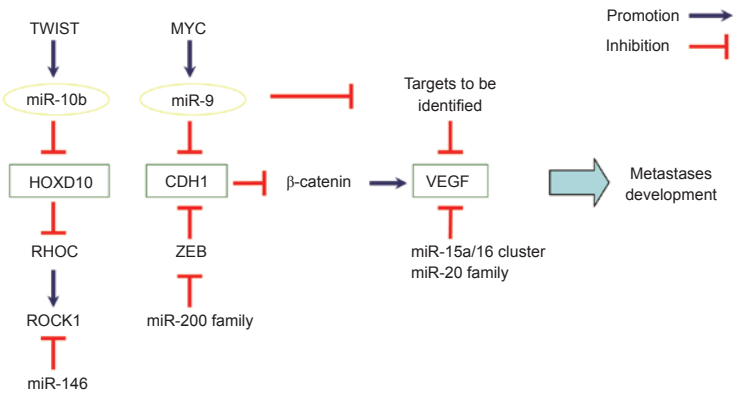

Figure 1 Two small non-coding RNAs (miR-9 and miR-10b) and the complex regulatory network of cancer metastasis (data from ref [2, 5, 6]).

In 2007, Ma and coworkers from Weinberg's group reported the first study about the role of miRNAs in tumor metastasis [5]. The authors identified three miRNAs, namely miR-155, miR-9 and miR-10b, as being upregulated in breast cancer cell lines and showed that miRNA-10b, by targeting HOXD10a, induce the pro-metastatic gene *RHOC* and consequently contribute to metastasis [5]. In a recent publication in *Nat Cell Biol* [6], Ma and colleagues elegantly show the involvement of miR-9 in the metastasis-regulating signaling network (Figure 1). A common approach to identify miRNA targets is the use of computational algorithms whereby the authors identify and further confirm by luciferase assay and western blot that the cell-cell adhesion receptor E-cadherin (encoded by *CDH1*) is a direct target of miR-9. This miRNA binds to CDH1 3′UTR region as a decrease in luciferase activity was detected in cells co-transfected with miR-9 and CDH1 3′UTR reporter gene. To further confirm this result, the authors show that co-transfection of miR-9 and CDH1 3′UTR reporter gene containing a mutation in the seed-target region do not cause a decrease in luciferase activity [6].

This finding is important because, as it has long been recognized, loss of E-cadherin expression is one of the hallmarks of EMT process [7]. E-cadherin is a transmembrane protein whose primary function is to mediate cell adhesion. The cytoplasmic tail of E-cadherin as-

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sociates with several proteins including β-catenin. Besides its involvement in cell adhesion, β-catenin is an essential effector of the Wnt signaling pathway which is frequently upregulated in tumors [8]. Specifically, β-catenin forms a transcription factor complex with TCF to regulate gene expression. When β-catenin escapes degradation, it accumulates in the cytosol and can enter the nucleus where it will act as a transcriptional co-regulator [7, 8]. miR-9 upregulation in breast cancer cells suppresses E-cadherin that consequently loses its capacity to sequester β-catenin and potentiates the Wnt signaling. In fact, the authors show that miR-9 expression increases β-catenin activity in cell lines with a basal E-cadherin expression. *In vivo*, miR-9 overexpressing tumor xenografts develop more micrometastasis than tumor xenografts without miR-9 overexpression. In addition, *in vivo*, inhibition of miR-9 can decrease metastasis formation [6]. Silencing of miR-9 and modulating E-cadherin expression may represent a new therapeutic approach in advanced breast cancers to prevent metastasis formation.

Study by Ma *et al.* is exciting because the authors demonstrate the contribution of miR-9 not only for EMT but also for induction of angiogenesis that is essential for metastasis to develop. VEGFA is a key pro-angiogenic protein and a target of β-catenin [9]. Ma *et al*. show that miR-9 overexpression upregulates VEGFA and that it correlates with E-cadherin expression. *In vivo*, the same result is obtained: VEGFA plasma levels in miR-9-overexpressing tumor xenografts are higher than in controls indicating the role of miR-9 in tumor angiogenesis. However, by itself E-cadherin and β-catenin do not induce VEGFA upregulation [6]. Therefore, other miR-9 targets have to be considered. In future studies it will be interesting to answer the question what other proteins/pathways may mediate VEGFA upregulation via miR-9 overexpression. Finally, Ma *et al*. explore upstream pathway of miR-9 expression regulation and find out its activation by MYC/MYCN. The authors correlate miR-9 upregulation with MYC amplification in neuroblastomas tumor samples and also with metastatic breast tumors [6]. However, the number of breast cancer samples used in the study is reduced and so it remains to be determined if miR-9 can be used as a prognostic marker since large breast cancer association studies are needed for this. In conclusion, this study convincingly identifies a new player in both EMT and angiogenic processes – miR-9, and opens intriguing new avenues of diagnostic and therapy: Do miR-9 levels in tumors and/or plasma of breast cancer patients predict metastasis? Should anti-miR-9 (and anti-miR-10b) be used for metastases prophylaxis in cancer patients?

Currently the role of miRNAs as one of key regulators of the metastatic process is starting to be clarified. Similar to miRNAs, other non-coding RNAs (ncRNAs) were shown to be deregulated in tumors [10]. In the future, it will be interesting to address the role of other ncRNAs in the complex molecular puzzle of metastasis.

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BRCA1, microRNAs and cancer predisposition: Challenging the dogma

Comment on: Pelletier C, et al. Cell Cycle 2011; 10:90–1

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Identifying breast cancer risk in BRCA1-negative (and BRCA2-negative) families is an important medical issue. *BRCA1* is a tumor suppressor gene that is important in the regulation of several cellular functions, such as DNA damage, the cell cycle, recruitment of chromatin-modifying proteins and ubiquitin ligase activity.[1] *BRCA1* mutations are well-known risk factors for developing breast cancer. These mutations may cause wild-type BRCA1 loss of function or create gain-of-function BRCA1-mutated proteins.[1] Although some researchers have focused their attention on understanding how *BRCA1* mutations affect cellular physiology and therefore affect cancer risk,[1] others have focused on finding new genetic markers in the *BRCA1* gene that can help predict breast cancer risk. However, less than 5% of all persons susceptible to breast cancer can be determined by analyzing *BRCA1* mutation status;[2,3] thus, new markers in the *BRCA1* gene must be identified. New players in the predisposition mechanism are microRNAs (miRNAs) **(Fig. 1**, see opposite page). This class of small, noncoding RNAs is approximately 22 nucleotides in length and can regulate gene expression post-transcriptionally by binding the 3′ untranslated region (UTR), the coding sequence or 5′UTR of target messenger RNAs (mRNAs), which can lead to inhibition of translation or mRNA degradation. It is already known that genetic variations in miRNAs or miRNA target sites can interfere with the miRNA-mRNA interaction, which then can affect expression levels of several proteins involved in cancer genesis and development, such as oncogenes and tumor suppressor genes (for a review see ref. 4).

Recently in *Cell Cycle*, Pelletier et al.[3] described rare *BRCA1* halotypes in the 3′UTR associated with breast cancer risk. The authors sequenced the *BRCA1* 3´UTR of breast cancer patients and found three previously reported and one novel single-nucleotide polymorphism (SNP). Using an outstanding normal population repository of 2250 individuals from 46 different geographic populations, the authors determined the variants' frequencies.

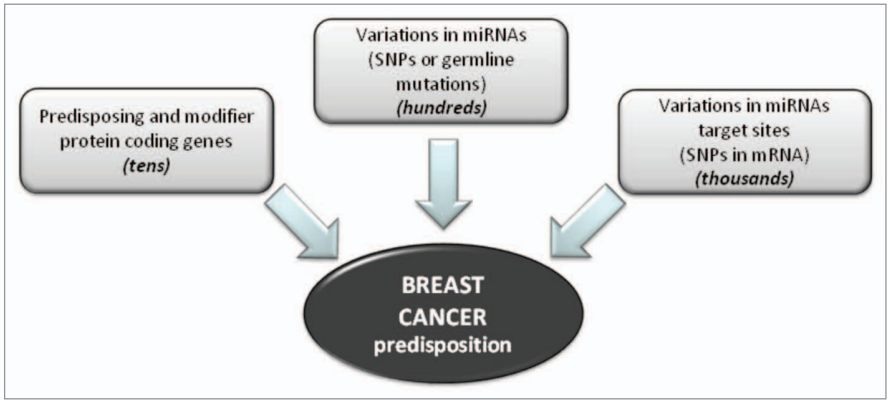

Figure 1. Challenging the dogma—miRNAs and cancer predisposition.

Pelletier et al. analyzed 8 SNPs and identified five haplotypes, three of which were located on the 3′UTR of *BRCA1*, that were present in the breast cancer population but rarely found in the control population. The frequency of these haplotypes also differed according to ethnicity and tumor subtype. Interestingly, these rare haplotypes were not associated with the most common *BRCA1*mutations and therefore might be independent markers of breast cancer risk. Given their potential as biomarkers, further study of the SNPs identified by Pelletier et al. is needed, particularly with respect to any disruptions in the interaction between miRNAs and mRNA that these variations might create.

Another example of a *BRCA1* SNP showing an association with breast cancer risk was reported by Nicoloso et al.[5] Those authors reported that the *BRCA1* SNP rs799917 is associated with susceptibility to breast tumor and that this risk is particularly increased for sporadic breast cancer. The authors showed that miR-638 interacts more strongly with allele [C] of SNP rs79991 than with the [T] allele. This difference in interaction was also confirmed at the protein level.[5]

In addition, miRNA-mRNA interactions dependent on SNPs have also been described in other tumor types. For instance, the laboratories of Slack and Weidhaas were the first to describe a SNP associated with non–small cell lung cancer risk in the *KRAS* 3′UTR that interferes with let-7 binding. The variant allele alters let-7-mediated regulation of KRAS, increasing its expression.[6]

These results demonstrate the importance of SNPs in conveying susceptibility to different kinds of cancer. The study by Pelletier et al.[3] is particularly vital because it describes new genetic markers in *BRCA1* 3′UTR noncoding regions that can improve our determination of breast cancer susceptibility. Inclusion of these SNPs in *BRCA1* haplotypes that are associated with breast cancer risk may guide future studies of functional miRNA interactions and their cellular consequences. This has the potential to greatly increase our ability to diagnose BRCA1-negative breast cancer at an earlier stage.

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Genetic variants at the miR-124 binding site on the cytoskeleton-organizing *IQGAP1* gene confer differential predisposition to breast cancer

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Abstract. *IQGAP1* knockout mice develop gastric cancer, but the IQGAP1 protein is associated with some advanced-stage human cancers. IQGAP1 expression is regulated by a microRNA, miR-124, through a binding site at the 3'-untranslated region, where a single nucleotide polymorphism (SNP) exists in the core binding region. We asked whether *IQGAP1* expression is associated with breast cancer development and whether genetic variants at the miR-124 binding site are important. We genotyped the *IQGAP1* SNP rs1042538 A/T in 1,541 breast cancer cases and 1,598 controls and analyzed the frequency of the variant and interactions with major risk factors in these populations. We also measured the expression of IQGAP1 at both mRNA and protein levels in different *IQGAP1* genotypes. The *IQGAP1* TT genotype, compared with the AA genotype, was associated with a significantly lower risk of developing breast cancer [P=0.049, odds ratio (OR), 0.78; 95% confidence interval (CI), 0.61-0.99]. In case-only analyses, the TT, compared with the AA, genotype was associated with progesterone receptor-positive subjects (OR, 1.35; 95% CI, 1.00-1.83). The expression levels of IQGAP1 protein were significantly higher in the TT genotype compated to the AA genotype. The presence of SNPs at the miR-124 binding site may be a marker for predicting breast cancer risk and prognosis.

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Key words: breast cancer, microRNA, SNP, *IQGAP1*, case-control study

Introduction

During embryonic development and throughout adult life in humans, gene expression is tightly regulated by a complex biological network in a tissue-specific manner and in close interaction with the environment to ensure accurate spatial and temporal differentiation of multiple organs with distinct functions. Significant deviation in the key regulators of this network via mutations often results in pathogenesis, including cancer. Subtle differences in the key regulatory genes via single nucleotide polymorphisms (SNPs) can result either in non-pathologic differences, such as hair color, or in increased propensity for a diseased state, such as cancer. Revealing the roles of these SNPs is a major area of research in the realm of molecular epidemiology in the era of postgenomic medicine (1).

In recent years, studies have revealed the importance of a class of small non-coding RNAs, microRNAs (miRNAs), that are critically involved in regulating gene expression (2). miRNAs directly regulate about 30% of the genes in the human genome via degradation or translational inhibition of their target messenger RNAs (mRNAs) and are thus important regulators of cellular processes such as differentiation, proliferation, mobility, and apoptosis (3,4). The first miRNA-target mRNA pair to be verified *in vivo* was *let-7* miRNA and its target, *lin-41*; and the natural interaction between this miRNA and its target is one of the best understood (5). miRNAs suppress gene expression mainly by binding to the complementary sequences in the 3 untranslated regions (UTRs) of mRNA of their target genes (6), although some miRNAs have been shown to act as an enhancer element to increase gene expression (7). It is generally believed that nucleotides 2-8 from the 5' end of miRNA, called the 'seed region', are the most critical for miRNA binding (8). Perfect Watson-Crick complementarity is observed in these 7 consecutive base pairs in most cases. Therefore, a single nucleotide change in this region may cause sufficient disruption in binding to deregulate the target genes (9). If the target gene is an oncogene, a tumor suppressor gene, or other regulatory genes that are critical for homeostasis, the single nucleotide change in the miRNA binding site may alter

the expression of these genes and shift the normal cellular program to a cancer-prone state, thus increasing cancer risk and/or conferring a specific cancer phenotype (10,11).

Breast cancer is the most common cancer among women in developed countries, and an alarming increase in incidence has been seen in developing countries (12). Germline mutations in *BRCA1* and *BRCA2* genes account for only 5% of all breast cancer cases in the general population (13,14). Other low-penetrance genetic variants, especially in as-yet unknown combinations, are expected to explain most breast cancer incidence (15). Investigators, including those in our group, have hypothesized that the 3' UTRs of miRNA target genes may harbor such important variants (10,16). Saunders *et al* (17) conducted a bioinformatic survey of the human genome for SNPs in putative miRNA target sites and found an appreciable level of variations within predicted as well as experimentally verified miRNA targets. One of the SNPs highlighted in the study of Saunders *et al* was SNP rs1042538 in the *IQGAP1* gene (encoding IQ motif-containing GTPase-activating protein 1), and this SNP has been experimentally verified to disrupt a miRNA target site sequence for miRNA-124 (miR-124) (18).

The scaffold protein IQGAP1 integrates signaling pathways and participates in diverse cellular activities that are important for both normal development and diseased states (19-24). IQGAP1 has attracted attention from cancer and developmental biologists because IQGAP1 expression appears to play an opposite role in normal development and in cancer progression. Studies with human tumor tissues have suggested that *IQGAP1* is an oncogene, which is overexpressed in a number of human solid neoplasms, including cancers of the colon, ovaries, stomach, and breast as well as glioblastoma (25-29). Functional studies have established the fact that IQGAP1 interacts with and regulates the actin-Cdc42/Rac1-mitogen-activated protein kinase pathway, thus contributing to its role in cell migration and invasion (19). Furthermore, another report suggested that IQGAP1 is involved in the expansion of cancer stem cells in glioblastoma and that together with IGFBP2 was associated with shorter survival in glioblastoma patients (27). Similarly, IQGAP1 was shown to be a marker of poor prognosis in ovarian cancer (26). In gastric cancer, it was shown that IQGAP1 was up-regulated by gene amplification (30).

However, the putative oncogenic properties of IQGAP1 have been contradicted by the finding that the deletion of *IQGAP1* in mice resulted in hyperplasia in gastric epithelial cells (31), suggesting that the *IQGAP1* gene plays an important role in normal development and actually has an antiproliferative function in normal epithelial cells. In other words, the *IQGAP1* gene may have a tumor suppression function in normal cells but may turn into an oncogene in tumor cells through an unknown mechanism. These conflicting properties of IQGAP1 suggest that IQGAP1 function may be dependent on developmental stage and cell type. However, determining the exact role and regulation of IQGAP1 in normal and cancer development will require more extensive studies, including population-based and functional studies.

A large number of SNPs have been found within the sequence of the *IQGAP1* gene, but SNP rs1042538 is recognized as the only one targeted by a miRNA with a high frequency of variation in Chinese population. This SNP (A/T variant) is one of the seven consecutive nucleotides corresponding to the seed region of miR-124 (Fig. 1). The A allele, together with the other six, form a perfect pairing with the seed, which is responsible for the down-regulation of IQGAP1 by miR-124 (17). Therefore, we conducted a case-control study to investigate whether this SNP at the miRNA-binding site on the IQGAP1 gene plays a role in breast cancer development and prognosis in a Chinese population.

Patients and methods

Patients and controls. Study patients were recruited from the Breast Cancer Research Center in Tianjin Medical University Cancer Hospital, and clinical information was acquired from the Tianjin Cancer Registry upon the approval of the Institutional Review Board (32). This study included 1,541 patients with newly diagnosed and histologically confirmed breast cancer, who were consecutively recruited between January 1, 2007 and February 28, 2008. The response rate of the eligible patients we recruited was ~95%.

We also recruited 1,598 cancer-free women (controls) during this study period who were genetically unrelated to one another and living in the nearby community. The response rate of the eligible controls who were approached for recruitment was ~90%. The controls were frequency-matched to the cases by age (±5 years).

After the study participants signed an informed consent form, they were interviewed for demographic data and information about major risk factors, including family history. For the cases, we also collected information about tumor features and disease severity, including morphologic characteristics, mean age at diagnosis, infiltrating/invasive ductal carcinoma (IDC) status, tumor size, presence of lymph node and/or organ metastasis, clinical stage, and estrogen receptor (ER) and progesterone receptor (PR) status. Each eligible subject donated 20 ml of blood, which was collected into heparinized tubes and used for biomarker assays, including DNA extraction and genotyping.

Genotyping. From each blood sample, a leukocyte cell pellet, obtained from the buffy coat by centrifugation of 1 ml of whole blood, was used for DNA extraction. Genomic DNA was isolated with the Qiagen DNA Blood Mini Kit (Qiagen Inc., Valencia, CA) according to the manufacturer's instructions. For genotyping the rs1042538 A/T SNP, both the amplifying primers and the TaqMan MGB probes were designed for the TaqMan SNP Genotyping assays (Applied Biosystems, Foster City, CA). More than 10% of the samples were randomly selected for repeated assays, and the results were 100% concordant.

Quantitative measurement of IQGAP1 mRNA and protein expression. Total RNA was isolated from 37 frozen breast cancer tissues from patients with known genotypes of *IQGAP1* (AA or TT), as determined from their blood samples. The extraction and purification of total RNA were performed with TRIzol reagent (Invitrogen) according to the manufacturer's instructions. RNA quality and concentration were determined with the Agilent 2100 Bioanalyzer (Agilent Technologies). Real-time quantitative polymerase chain reaction (PCR) was performed in a 96-well reaction plate (MicroAmp® Optical

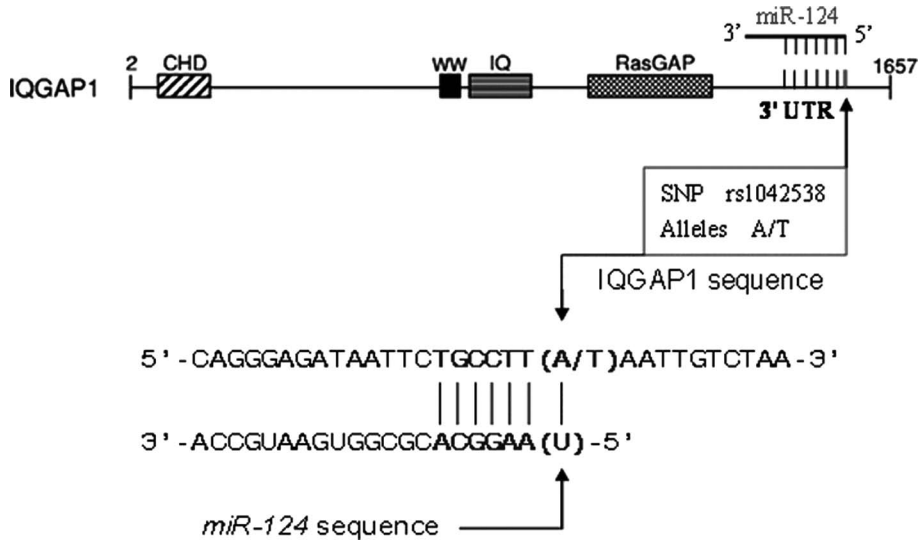

Figure 1. The base-pairing of the miR-124 seed region and the 3'-UTR of the *IQGAP1* gene, including the rs1042538 polymorphism. SNP rs1042538 has an A/T variation, which is one of the seven consecutive nucleotides corresponding to the seed region of miR-124. The A allele, together with the other six, forms a perfect pairing with the seed, which is responsible for the down-regulation of IQGAP1 by miR-124; the T allele forms a non-perfect pairing with the seed and may escape the regulation of miR-124.

96-Well Reaction Plate, Applied Biosystems) on an ABI PRISM® 7500 Sequence Detector System (Applied Biosystems), according to the manufacturer's instructions.

RT-PCR for IQGAP1 expression was done using power SYBR Green one-step RT-PCR master mix reagent kit (P/N 4391178). All primers were synthesized by Sangon Corp. (Shanghai, China). In order to normalize the differences in the amount of total RNA used in each reaction, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) RNA was measured as endogenous control. Each sample was analyzed in duplicate and the coefficient of variation of all reactions was <5%. The relative expression level of IQGAP1 to GADPH was described using the equation: expression = $2^{-\Delta Ct}$. Western blotting was done to evaluate the effect of miR-124 on the expression of IQGAP1 in cell lines, MM231 and LN299, using actin as a control. IQGAP1 protein expression was measured through Western blotting using actin as a loading control in 48 breast cancer tissues with IQGAP1 AA or TT genotype.

Statistical analyses. We used the χ^2 test to compare differences in frequency distributions of demographic variables, risk factors, and alleles of the *IQGAP1* polymorphism between the cases and controls. We also tested the Hardy-Weinberg equilibrium of genotype distributions in the controls. In addition, we used unconditional univariate and multivariate logistic regression analyses to examine the association between the SNP and breast cancer risk by estimating odds ratios (ORs) and 95% confidence intervals (CIs) with and without adjustment for age and other risk factors. Finally, we stratified the genotype data according to age, family history, and clinical variables (including morphologic characteristics, tumor size, presence of lymph node and/or organ metastases, tumor stage, and ER and PR status) of breast cancer patients by using the χ^2 test and logistic regression. The expression levels of IQGAP1 between AA and TT genotypes were compared with use of the rank-sum test. All statistical analyses were two-sided and performed with use of SAS software (version 9.0; SAS Institute, Cary, NC), and a P=0.05 was considered statistically significant.

Results

Case-control analysis of the IQGAP1 SNP. This case-control study included 1,541 breast cancer cases and 1,598 controls, and the distributions of known risk factors between cases and controls are shown in Table I. Age was adequately matched between cases and controls (P=0.242).

Genomic DNA for *IQGAP1* SNP genotyping was isolated from the peripheral blood of all participants with use of the TaqMan assay. The distribution of the *IQGAP1* genotypes (rs1042538) is shown in Table II. The SNP was in Hardy-Weinberg equilibrium in controls (P=0.469). The AA genotype was found in 33.42% of cases and 32.17% of controls, whereas the TT genotype was found in 17.26% of cases and 19.52% of controls. The TT genotype, compared with the AA genotype, was associated with a significantly lower risk of developing breast cancer (P=0.049, OR, 0.78; 95% CI, 0.61-0.99). In other words, women with the AA genotype were more likely than those with the TT genotype to develop breast cancer.

We also separated the cases into those with IDC (about 2/3 of all cases) and others (mainly including adenocarcinoma, carcinoma simplex, mucinous carcinoma, medullary carcinoma, lobular carcinoma). The two groups of cases were used in case-control analyses, and results revealed that in non-IDC cases only, those with the TT genotype had a significantly lower risk of developing cancer (OR=0.69; 95% CI, 0.50-0.94) than did those with the AA genotype (Table II). Thus, it appears that the effect of the IQGAP1 SNP is less apparent in more aggressive and invasive stages of breast cancer, when many genetic and epigenetic factors become involved.

Case-only analysis of IQGAP1 genotypes. The patient data we collected included information on mean age at diagnosis,

Table I. Frequency distributions of selected variables in breast cancer cases and cancer-free controls in Chinese women.

Variables	No. (%) of subjects		OR (95% CI)	P-value[a]
	Cases (n=1,541)	Controls (n=1,598)		
Age (years)				
≤50	739 (47.96)	733 (45.87)		0.242
>50	802 (52.04)	865 (54.13)		
Frequency of pregnancy				
≤2	710 (46.07)	728 (45.56)	1.00	0.771
>2	831 (53.93)	870 (54.44)	0.94 (0.80-1.11)	
Duration of breastfeeding (months)				
≤12	733 (47.57)	604 (37.80)	1.00	<0.001
>12	808 (52.43)	994 (62.20)	0.66 (0.56-0.77)	
Menopause[c]				
No	731 (47.65)	706 (44.54)	1.00	0.081
Yes	803 (52.35)	879 (55.46)	0.81 (0.64-1.03)	
Oral contraception[c]				
Never	1,197 (81.87)	1,321 (84.46)	1.00	0.057
Ever	265 (18.13)	243 (15.54)	1.14 (0.93-1.41)	
Smoking status[c]				
Never	1,309 (87.85)	1,478 (93.25)	1.00	<0.001
Ever	181 (12.15)	107 (6.75)	2.23 (1.70-2.93)	
Benign breast disease[c]				
Never	1,129 (73.79)	1,465 (92.78)	1.00	<0.001
Ever	401 (26.21)	114 (7.22)	4.41 (3.49-5.58)	
Family history of cancer[b,c]				
No	1,061 (68.90)	1,409 (88.45)	1.00	<0.001
Yes	479 (31.10)	184 (11.55)	3.24 (2.65-3.95)	

CI, confidence interval; OR, odds ratio. [a]Two-sided χ^2 test. [b]First- and second-degree relatives. [c]Due to missing values, the number of cases and controls are less than 1,541 and 1,598, respectively.

IDC status, tumor size, presence of lymph node and/or organ metastasis, clinical stage, and ER/PR status. Our analysis did not reveal association between any genotypes and mean age at diagnosis (P=0.387), IDC status (IDC vs. non-IDC, P=0.194), tumor size (P=0.821), lymph node metastases (P=0.652), clinical stage (P=0.773), or ER status (P=0.386) (Table IV). Our analysis revealed significant association between genotypes and PR status of cases. Specifically, cases with the TT genotype were more likely to be PR-positive than were those with the AA genotype (OR, 1.35; 95% CI, 1.00-1.83) (Table III). Furthermore, in ER-negative cases, the IQGAP1 genotype was significantly associated with PR status. Cases with AT or TT genotypes, compared with the AA genotype, were more likely to be PR-positive (P=0.022) (OR for AT genotype, 1.50; 95% CI, 1.01-2.23; OR for TT genotype, 1.91; 95% CI, 1.18-3.09) (Table IV).

Analysis of IQGAP1 expression. Our hypothesis was that IQGAP1 TT genotype, evading the regulation of miR-124, would have a higher level of IQGAP1 expression. We thus measured IQGAP1 expression at mRNA and protein levels using breast cancer tissue. IQGAP1 mRNA was higher in AA genotype than in TT genotype (0.040 vs. 0.025), the difference between the AA genotype and the TT genotype was not significant (rank-sum test, P=0.109) (Fig. 2A). The relative IQGAP1 protein levels were higher in TT genotype than in

Table II. Multivariate-adjusted ORs for risk of breast cancer associated with the *IQGAP1* polymorphism.

Genotype	No. (%) of all subjects			No. (%) of patients with IDC			No. (%) of patients with other morphology		
	Cases (n=1,541)	Controls (n=1,598)	OR[a] (95% CI)	Cases (n=1,065)	Controls (n=1,598)	OR[a] (95% CI)	Cases (n=476)	Controls (n=1,598)	OR[a] (95% CI)
IQGAP1 (A/T)									
AA	515 (33.42)	514 (32.17)	1.00	345 (32.39)	514 (32.17)	1.00	170 (35.71)	514 (32.17)	1.00
AT	760 (49.32)	772 (48.31)	0.96 (0.79- 1.15)	525 (49.10)	772 (48.31)	1.01 (0.85-1.21)	235 (49.37)	772 (48.31)	0.92 (0.73-1.16)
TT	266 (17.26)	312 (19.52)	**0.78 (0.61-0.99)**[b]	195 (18.31)	312 (19.52)	0.93 (0.74-1.17)	71 (14.92)	312 (19.52)	**0.69 (0.50-0.94)**[b]
Trend test			P=0.160			P=0.604			P=0.026

CI, confidence interval; OR, odds ratio. [a]ORs are adjusted for age, duration of breastfeeding, menopause, oral contraception, smoking status, benign breast disease, and family history of cancer. [b]Statistically significant.

AA genotype (125.46 vs. 51.35), the difference between the TT genotype and the AA genotype was statistically significant (rank sum test, P=0.039) (Fig. 2B).

Discussion

We began this case-control study to find an answer to the question of whether the SNP at the miR-124 binding site on the 3'-UTR of the cytoskeleton-organizing gene *IQGAP1* had any effect on breast cancer risk. We did not have an a prior prediction because the published results on the function of IQGAP1 appeared to be contradictory. On the one hand, removal of the *IQGAP1* gene predisposed mice to the development of gastric cancer. On the other hand, IQGAP1 expression was elevated in many cancers and in some tumors correlated with poor prognosis. Our case-control study showed that the AA genotype of the miR-124 binding site SNP on *IQGAP1*, which renders its down-regulation by miR-124, was associated with increased risk of breast cancer. Although this does not prove that IQGAP1 plays a role in tumor suppression, the result is consistent with the knockout mouse studies that suggest that IQGAP1 is a negative regulator for cancer development. We also examined IQGAP1 expression in breast cancer tissues and their adjacent pathologically normal tissues and found that indeed, IQGAP1 was up-regulated in breast cancer tissue, similar to findings in other previously reported studies (33). Moreover, IQGAP1 knockdown experiments showed that reduction of IQGAP1 resulted in increased cell growth in non-cancer breast epithelial cells but resulted in decreased cell growth in breast cancer cells, supporting that IQGAP1 plays an opposite role in normal development and cancer. Or in other words, IQGAP1 may indeed act as a tumor suppressor in breast cancer initiation but act as an oncogene after cancer has developed. Therefore, our findings may help explain the apparently contradictory results regarding IQGAP1 in the same human populations, at least partially removing the argument that IQGAP1 may work differently in humans and mice.

The A allele, which binds perfectly with miR-124 and leads to the down-regulation of IQGAP1 expression, is associated with increased risk of breast cancer. This finding suggests that IQGAP1, and by extension cytoskeleton organization, is critical for normal breast cell development. A lack of IQGAP1 and disrupted cytoskeleton organization contributed to breast cancer development or hyperplasia in gastric epithelial cells in the mouse studies (31), although it is not known whether there were any abnormalities in breast epithelial cells in the *IQGAP1* gene knockout mice or whether the A allele of our studied SNP had any effect on gastric cancer development. Nevertheless, because cytoskeleton structure is critical for maintaining highly organized and polarized epithelia cell sheets, the requirement for sufficient IQGAP1 expression is quite logical. Further case-control studies are clearly warranted in other types of cancer.

Our population-based case-control study suggests that the T allele of the miR-124 binding site SNP on the IQGAP1 gene is a 'good' variant that has a breast cancer protection function. Of interest, the T allele was found in frequencies of 40, 10, and 10%, respectively, for the HapMap panels of Asian, European, and African women. It is well established that breast cancer

Table III. Association of the *IQGAP1* polymorphism with clinical characteristics of breast cancer cases.

Variables	No. of patients	IQGAP1 rs1042538 (A/T) genotype frequency			P-value[a]
		AA (n=515)	AT (n=760)	TT (n=266)	
Age at diagnosis (years)	1,540[b]	51.61±10.50	52.13±11.11	51.14±10.18	0.387
Morphology	1,541				
IDC		345 (66.99)	525 (69.08)	195 (73.31)	0.194
Others		170 (33.01)	235 (30.92)	71 (26.69)	
OR (95% CI)		1.00	0.91 (0.72-1.15)	0.74 (0.53-1.03)	
Tumor size	1,395[b]				
≤2 cm		171 (36.93)	242 (35.12)	87 (35.80)	0.821
>2 cm		292 (63.07)	447 (64.88)	156 (64.20)	
OR (95% CI)		1.00	1.08 (0.85-1.38)	1.05 (0.76-1.45)	
Lymph node metastases	1,534[b]				
No		309 (60.47)	473 (62.48)	159 (59.77)	0.652
Yes		202 (39.53)	284 (37.52)	107 (40.23)	
OR (95% CI)		1.00	0.92 (0.73-1.16)	1.03 (0.76-1.39)	
Clinical stage	1,394[b]				
0+I		122 (26.41)	193 (27.97)	63 (26.03)	0.773
II+III+IV		340 (73.59)	497 (72.03)	179 (73.97)	
OR (95% CI)		1.00	0.92 (0.71-1.21)	1.02 (0.72-1.45)	
ER	1,515[b]				
-		224 (44.09)	314 (42.32)	125 (47.17)	0.386
+		284 (55.91)	428 (57.68)	140 (52.83)	
OR (95% CI)		1.00	1.08 (0.86-1.35)	0.88 (0.66-1.19)	
PR	1,515[b]				
-		241 (47.44)	314 (42.32)	106 (40.00)	0.085
+		267 (52.56)	428 (57.68)	159 (60.00)	
OR (95% CI)		1.00	1.23 (0.98-1.54)	**1.35 (1.00-1.83)**[c]	

CI, confidence interval; ER, estrogen receptor; IDC, infiltrating ductal carcinoma; OR, odds ratio; PR, progesterone receptor. [a]Two-sided χ^2 test for difference in frequency distribution of variables between *IQGAP1* genotypes. [b]Due to missing values, n is <1,541. [c]Statistically significant.

incidence is much lower among Asian women than among women of European or African descent. Such differences have often been attributed to different lifestyles in the absence of known genetic factors. However, if the T allele is indeed protective, this allele and other similar alleles may, at least in part, constitute the genetic basis for the varying incidence in breast cancer among Asian, European, and African women. Future population-based studies in European and African women cohorts will be needed to test this hypothesis.

In our study, we also evaluated the potential association of this SNP with breast cancer prognosis. This was because IQGAP1 has been shown to be overly expressed in cancer, and expression levels have been associated with poor prognosis in several other cancer types, including glioblastoma (34). This association is not surprising because enhanced cell migration, invasion, and metastasis of cancer cells require heightened cytoskeleton reorganizing activities. However, we were initially surprised with our analysis results that showed a lack of correlation of this SNP with major clinical parameters such as metastasis and tumor invasiveness in our breast cancer cases. We did observe some association with PR status, but the significance of this association is currently unclear. A few

Table IV. Association of the *IQGAP1* polymorphism with clinical characteristics of breast cancer in estrogen receptor-negative cases.

Variables	No. of patients	IQGAP1 rs1042538 (A/T) genotype frequency			P-value[a]
		AA (n=224)	AT (n=314)	TT (n=125)	
Tumor size	603[b]				
≤2 cm		64 (32.00)	97 (33.68)	36 (31.30)	0.873
>2 cm		136 (68.00)	191 (66.32)	79 (68.70)	
OR (95% CI)		1.00	0.93 (0.63-1.36)	1.03 (0.63-1.69)	
Lymph node metastases	663				
No		129 (57.59)	199 (63.38)	78 (62.40)	0.381
Yes		95 (42.41)	115 (36.62)	47 (37.60)	
OR (95% CI)		1.00	0.79 (0.55-1.11)	0.82 (0.52-1.28)	
Clinical stage	603[b]				
0+I		41 (20.60)	74 (25.61)	27 (23.48)	0.441
II+III+IV		158 (79.40)	215 (74.39)	88 (76.52)	
OR (95% CI)		1.00	0.75 (0.49-1.16)	0.85 (0.48-1.47)	
Morphology	663				
IDC		158 (70.54)	223 (71.02)	93 (74.40)	0.721
Others		66 (29.46)	91 (28.98)	32 (25.60)	
OR (95% CI)		1.00	0.98 (0.67-1.42)	0.82 (0.50-1.35)	
PR	662[b]				
-		173 (74.76)	217 (70.21)	80 (63.30)	0.022
+		51 (25.24)	96 (29.79)	45 (36.70)	
OR (95% CI)		1.00	**1.50 (1.01-2.23)**[c]	**1.91 (1.18, 3.09)**[c]	

CI, confidence interval; IDC, infiltrating ductal carcinoma; OR, odds ratio; PR, progesterone receptor. [a]Two-sided χ^2 test for difference in frequency distribution of variables between *IQGAP1* genotypes. [b]Due to missing values, n is <663, the total number of patients with AA, AT, or TT genotypes. [c]Statistically significant.

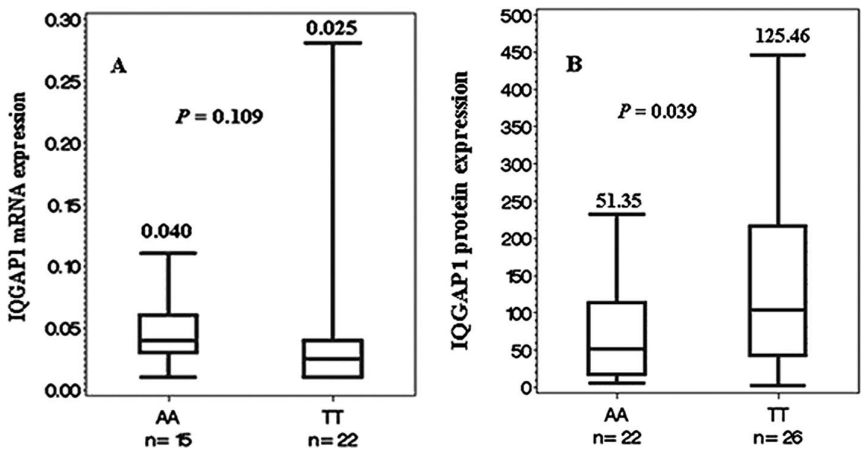

Figure 2. IQGAP1 expression in tumor tissue with IQGAP1 AA or TT genotypes. (A) Comparison of IQGAP1 expression at mRNA level between IQGAP1 genotypes (AA and TT) in breast cancer tissue. The difference of IQGAP1 mRNA between the TT genotype and the AA genotype was not significant (rank-sum test, P=0.109). (B) Comparison of IQGAP1 expression at protein level between IQGAP1 genotypes (AA and TT) in breast cancer tissue. IQGAP1 protein was higher in TT genotype than in AA genotype, the difference between the TT genotype and the AA genotype was statistically significant (rank-sum test, P=0.039).

other studies have also shown an association between genetic variations and PR status. In a case-control study with more than 9,000 subjects, Pooley *et al* (35) found that a coding SNP (rs3218536) in one of the DNA double-strand break repair genes, *XRCC2*, was strongly associated with risk of developing PR-positive breast cancer. In another study, Synowiec *et al* (36) reported an association between the *RAD51*-135G/C polymorphism and PR expression (OR=6.33; 95% CI=1.15-35.01).

Our results suggest a number of possibilities. First, IQGAP1 expression in breast cancer may not be strongly regulated by miR-124, and thus the SNP site for miR-124 binding may be irrelevant. Although we do not have direct evidence to support this possibility, one study found that miR-124 promoter is methylated in acute lymphoblastic leukemia (ALL) and that the expression of miR-124 in ALL is very low (37). Our analysis of miR-124 expression supported this observation that miR-124 is decreased in cancer tissue compared with adjacent normal tissue. A second scenario is that IQGAP1 expression levels may be less important to breast cancer because other genes in the same pathway are more activated. If this is true, then the expression of IQGAP1 should not be correlated with prognosis of breast cancer. We tested this hypothesis by examining the gene expression profile data in the public domain and showed that indeed, IQGAP1 expression levels in two breast cancer datasets did not correlate with grade or metastasis. We also showed that IQGAP1 expression did not correlate with grade in gastric cancer. In contrast, IQGAP1 expression levels were associated with grade and poor survival in gliomas, consistent with the literature.

In this study, we could not evaluate the association between IQGAP1 SNP genotypes and breast cancer survival, mainly because the breast cancer cases recruited for our study were relatively recent, which meant that there was not enough follow-up time for analysis of survival or distant metastasis. However, in the van't Veer *et al* (38) study, clinical follow-up of 78 breast cancer patients for more than 5 years after lumpectomy showed that IQGAP1 expression levels were not correlated with distance metastasis. Therefore, the role of IQGAP1 in cancer prognosis is apparently dependent on cancer type.

In conclusion, we have conducted the first epidemiologic study of the association between the *IQGAP1* SNP within the miR124 binding site and breast cancer risk. Our study showed that the regulation of IQGAP1 could be complex and that the role of IQGAP1 in cancer cannot be generalized. However, the role of this SNP in cancer risk may be broader because of the different frequency distributions of demographic variables, risk factors, and alleles of the IQGAP1 polymorphism between Chinese women and women of other ethnic groups. Because of its potential significance, this study should be replicated with populations of various ethnic backgrounds. Once validated, this SNP may be important for genetic testing and screening of individuals at high risk of breast cancer.

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Mini review

MicroRNA history: Discovery, recent applications, and next frontiers

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ABSTRACT

Since 1993, when the first small non-coding RNA was identified, our knowledge about microRNAs has grown exponentially. In this review, we focus on the main progress in this field and discuss the most important findings under a historical perspective. In addition, we examine microRNAs as markers of disease diagnosis and prognosis, and as new therapeutic targets.

1. Introduction

MicroRNAs (miRNAs) are small non-coding RNAs (ncRNAs), approximately 20 nucleotides (nt) in length, that regulate gene expression posttranscriptionally by binding to 3′untranslated regions (UTR), coding sequences or 5′UTR of target messenger RNAs (mRNAs), and leading to inhibition of translation or mRNA degradation [1–3]. It is estimated that miRNAs regulate approximately 30% of the human protein-coding genome [1]. miRNAs control the expression of genes involved in several biologic processes, including apoptosis, proliferation, differentiation, and metastasis [1–3].

Two decades ago, both the existence and the importance of miRNAs were completely unknown. Until then, the scientific community focused on genes that codify for protein. The classical dogma that DNA is transcribed into RNA, which then is translated into protein, pushed aside the study of all the non-protein-coding sequences. Only in 1993 did the importance of miRNAs begin to be revealed [4,5]. This review will highlight the pioneer studies that have contributed to the brief history of miRNAs (Fig. 1).

2. Discovery of the first miRNA: *lin-4*

Lin-4 was the first miRNA to be discovered, in 1993, by the joint efforts of Ambros's and Ruvkun's laboratories [3,4]. In the nematode *Caenorhabditis elegans*, heterochronic genes control the temporal development pattern of all larval stages. One of these genes is *lin-4*, discovered by the isolation of a null mutation that causes a failure in temporal development [6,7]. Animals with lin-4 loss-of-function mutations are missing some adult structures, are incapable of laying eggs, and reiterate early development programs at inappropriate late larval stages. Lin-4 activity is required for the transition from the L1 to L2 stage of larval development [7,8]. In 1987, Ferguson et al., at Horvitz's laboratory, found that a suppressor mutation in the gene *lin-14* was able to revert the *null-lin-4* mutation phenotype [9]. In fact, null mutations in *lin-14* gene caused an exactly opposite phenotype of the *null-lin-4* mutations [8,9]. This interesting opposite phenotype between defects in *lin-4* and *lin-14* genes indicated that lin-4 could negatively regulate lin-14 [8]. In 1989, Ambros worked with Ruvkun, at Horvitz's laboratory, to clone the lin-14 gene [8,10]. At this time, the two colleagues followed two independent research careers, with Ambros focusing on the *lin-4* gene and Ruvkun on the *lin-14* gene.

Ambros, together with Lee and Feinbaum, found that a 700-bp fragment could contain *lin-4* gene but could not find the conventional start and stop codons. Even so, they introduced mutations in the putative open reading frame but lin-4 function remained unchanged. Therefore, Ambros concluded that *lin-4* did not encode a protein [4,8]. In addition, they found two very small lin-4 transcripts of only 61 nt and 22 nt in length [4]. On the other hand, Ruvkun and his colleagues Wightman and Ha found that *lin-14* was downregulated at a posttranscriptional level and that the *lin-14* 3′UTR region was sufficient for the temporal regulation [5]. The two groups shared their unpublished data, and in June 1992 Ambros and Ruvnkun independently came to the same conclusion: *lin-4* transcripts were complementary to a repeated sequence in the 3′UTR of the *lin-14* gene [4,5,8]. In December 1993, Ambros and Ruvkun independently reported in the same issue of *Cell* that the small and non-protein-coding transcript *lin-4* regulates *lin-14* through its 3′

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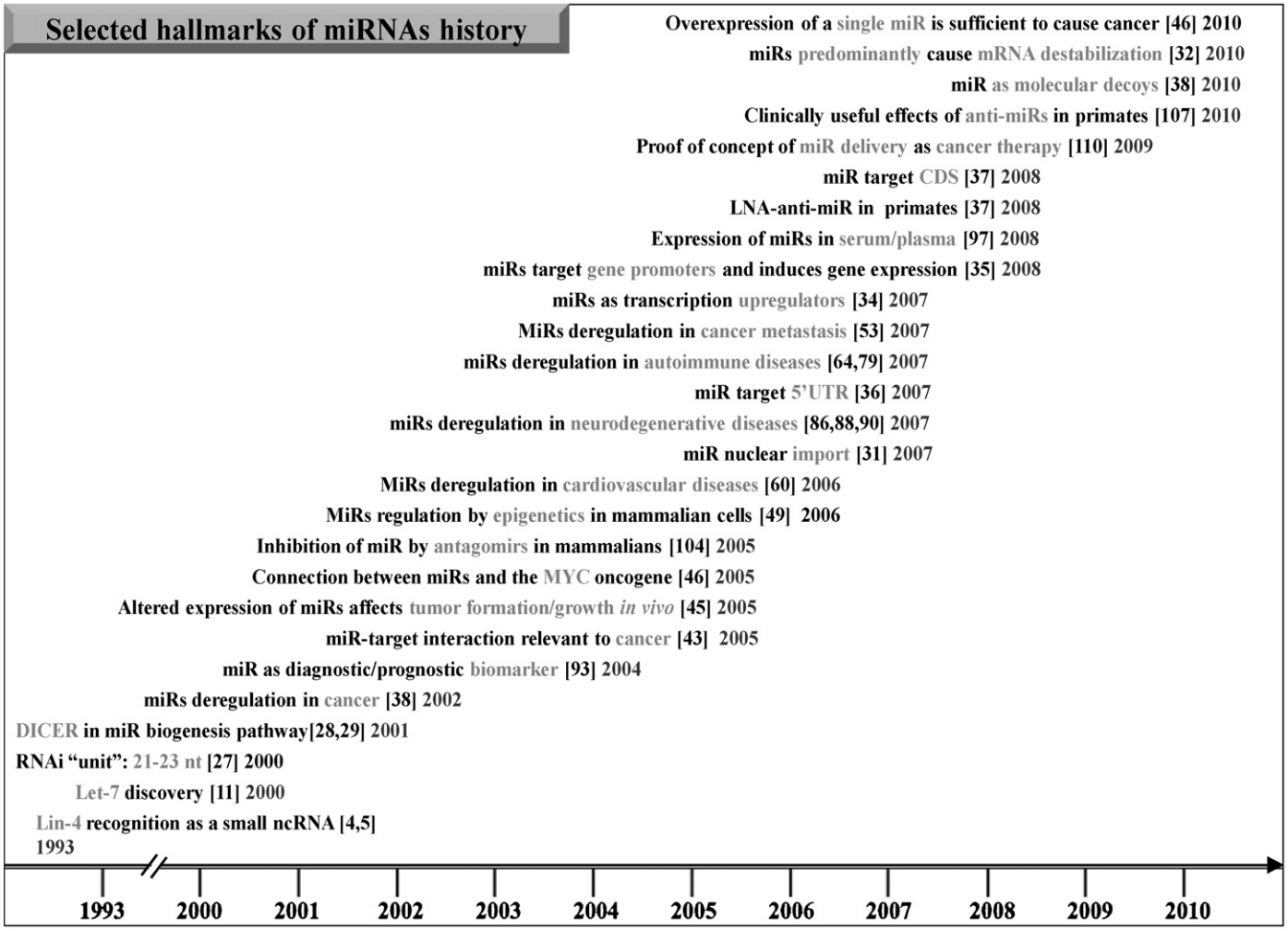

Fig. 1. Historical perspective on the evolution of our knowledge about miRNAs. miR: microRNA; UTR: untranslated regions; CDS: coding sequences; LNA: locked nucleic acid.

UTR region [4,5,8]. A new unexpected cellular regulatory mechanism involving a non-protein-coding transcript had been found!

3. Discovery of a second microRNA: Let-7

Likewise lin-4, let-7 is a heterochronic gene of *C. elegans* and was the second miRNA to be discovered, in 2000, seven years after the finding of the first miRNA. Reinhart et al. at Ruvkun's laboratory reported that *let-7* was a 21-nt RNA controlling the L4-to-adult transition of larval development [11]. Loss of let-7 activity causes reappearance of larval cell fates during the adult stage of development, while increased let-7 activity causes precocious expression of adult fates [11]. Remarkably, the authors found that the retarded let-7 phenotype could partially suppress *lin-41* (a *let-7* target) loss-of-function mutations [11]. In fact, *let-7* is complementary to two closely spaced sites in *lin-41* 3′UTR. Deletion of the *lin-41* 3′UTR region and *let-7* mutations abolish *lin-41* downregulation, showing that both partners are necessary for this mechanism [11–13].

Let-7 controls late temporal transitions during development across animal phylogeny. Unlike *lin-4*, the *let-7* sequence is conserved across species from flies to humans [14], a fact that had a major effect on the study of miRNAs in other organisms [15,16]. *let-7* RNA was detected in vertebrate, ascidian, hemichordate, mollusk, annelid, and arthropod but not in RNAs from plant and unicellular organisms [14,16]. In humans, it was detected at different expression levels in the majority of the tissues, including brain, heart, kidney, liver, lung, trachea, colon, small intestine, spleen, stomach, and thymus [14]. The *let-7* miRNA family refers to miRNAs that share complete sequence identity with *let-7* at the 5′ ends, termed seed regions, and therefore can regulate the same targets [15]. The let-7 family within humans comprises 12 miRNAs. Some members of the let-7 family identified and functionally analyzed in *C. elegans* include miR-48, miR-84, and miR-241 [17–19]. These miRNAs act redundantly to control the L2-to-L3 transition, by repressing *hbl-1* [19]. Functional cooperation among miRNA family members continues to be studied [20].

The discovery that let-7 is conserved across species triggered a revolution in the research of a new class of small ncRNAs, called miRNAs. Currently, thousands of miRNAs have been identified in humans and other species, and miRNA online sequences repositories, such as the miRbase database, are available [21–23]. Furthermore, current tools and software developed for miRNA target prediction facilitate studies of miRNAs functional network [24,25].

4. miRNA biogenesis pathway and miRNAs function

miRNAs are encoded in the genome as long primary transcripts (named pri-miRNAs) that contain a cap structure at the 5′ end and are poly-adenylated at the 3′ end. Pri-miRNAs are processed by the cellular RNaseII enconucleaseIII Drosha, together with DGCR8/Pasha proteins, into a structure of 60–110 nt, called

precursor-miRNA (pre-miRNA), which is then exported from the nucleus to the cytoplasm by an Exportin-5-dependent mechanism. In the cytoplasm, the pre-miRNA is cleft by the RNaseIII enzyme Dicer-1, together with TRBP/PACT proteins, producing a short imperfect double-stranded miRNA duplex. This duplex is then unwound by an helicase into a mature miRNA, approximately 20 nt in length, which is then incorporated into a multicomponent complex constituted by Argonaute family protein members known as RISC [26].

In a historical perspective, the knowledge acquired from RNAi was determinant for understanding miRNAs processment and activity. In 2000, Zamore et al. studied the RNAi process and found that double-stranded RNA fragments of 21–23 nt were targeting the mRNA cleavage [27]. The functional unit of RNAi was therefore the same size as miRNAs. In 2001, two reports were crucial for elucidation of the miRNA biogenesis mechanism, as both reports suggested an involvement of RNAi pathway components in miRNAs maturation [28,29]. Grishok and colleagues showed that a homologue of *Drosophila* Dicer (*dcr-1*) and two homologues of rde-1 (*alg-1* and *alg-2*) were essential for lin-4 and let-7 activity. Inactivation of these genes caused phenotypes similar to *lin-4* and *let-7* mutations [28]. Simultaneously, Hutvágner et al. found that let-7 pre-miRNA is cleaved by Dicer. In fact, when cells were transfected with siRNA duplex corresponding to human Dicer enzyme, pre-let-7 accumulated in the cells [29]. Taken together, these two studies corroborated the intersection between RNAi and miRNA pathway and opened the door for understanding the formation of mature miRNAs.

In the miRNA biogenesis pathway, Drosha and Dicer are spatially separated, being localized in the nucleus and the cytoplasm, respectively. In 2004, Lund et al. reported that Exportin-5 was the key player mediating the efficient nuclear export of the short miRNA precursors [30]. However, some miRNAs such as miR-29b are predominantly located in the nucleus [31]. In 2007, Hwang et al. found that some miRNAs contain additional sequence elements that control their subcellular localization. In fact, miR-29b possesses a hexanucleotide terminal motif that directs its import into the nucleus. These authors showed that, despite the small size of miRNAs, they can contain additional cis-acting regulatory motifs that might influence their posttranscriptional behavior, and they concluded that miRNAs with common 5′ ends, predicted to regulate the same targets, might have distinct functions [31].

miRNAs' main function is to inhibit protein synthesis of protein-coding genes, either by inhibition of translation or mRNA degradation. However, the relative contribution of each mechanism to repression was unknown until recently. In an elegant study, Guo et al. used ribosome profiling to measure the overall effects on protein production and simultaneously measured effects on mRNA levels. They concluded that inhibition of translation (no changes in mRNA levels of miRNA targets) had a modest influence on repressing protein levels, whereas mRNA destabilization was the predominant miRNAs mechanism of action to decrease their targets levels [32].

In addition to mRNAs repression, miRNAs have been also reported to activate translation of targeted mRNAs [33,34]. Vasudevan et al. were the first to clearly demonstrate that, in some instances, miRNAs can work as translational activators. TNFα AU-rich elements recruited miR-369-3 to mediate translation upregulation, exclusively under serum starvation conditions. In addition, upon cell cycle arrest, let-7 and the synthetic miRNA-cxcr4 induced translation, whereas they repressed translation in proliferating cells. Therefore, miRNAs can switch between translation repression and activation in coordination with the cell cycle [34]. In 2008, Place et al. provided new evidence that miRNAs can induce gene expression and were the first to show that miRNAs can target gene promoters. These authors showed that miR-373 targets the promoter of E-cadherin and CSDC2 and induced their expression [35].

For a long time, studies on miRNA-target interaction were confined to 3′UTR of mRNAs, probably because the first studies on miRNAs focused on this region. In 2007, Lytle et al. were the first to suggest that miRNAs could associate to any position of target mRNAs and demonstrated that mRNA targets were efficiently repressed by miRNA-binding sites in 5′UTR [36]. In 2008, Tay et al. reported that binding sites in coding sequences are abundant and experimentally showed that mouse *Nanog*, *Oct4*, and *Sox2* genes have miRNA-binding sites in their coding sequences. MiRNAs targeting these genes modulate embryonic stem cell differentiation [37].

In 2010, Eiring et al. reported a remarkable finding for our understanding of how miRNAs function. These authors found that, in addition to miRNAs gene silencing activity through base pairing with mRNA targets, miRNAs also have decoy activity that interferes with the function of regulatory proteins [38]. In particular, miR-328 binds to hnRNP E2 independently of the miRNA's seed region and prevents its interaction with *CEBPA* mRNA [38]. In conclusion, these authors introduced the new concept that miRNAs can work as molecular decoys for RNA-binding proteins [38].

5. MicroRNAs in disease: an historical perspective

5.1. MicroRNAs in cancer

The first report suggesting a role of miRNAs in cancer was published in 2002 [38]. *MiR-15* and *miR-16* were found to be located at chromosome 13q14, a region frequently deleted in chronic lymphocytic leukemia (CLL). Calin et al. discovered that both genes were deleted or downregulated in greater than 60% of B-cell human CLL, indicating that these genes behave as tumor suppressors in CLL [39]. Consequently, the same group found that a significant percentage of miRNAs is located at fragile sites and in regions altered in cancers, including regions of amplification or loss of heterozygosity or breakpoints, suggesting that miRNAs as a new class of genes had a relevant role in human cancer pathogenesis [40].

Oligonucleotide miRNA microarrays and, more recently, deep sequencing (next generation sequencing) have permitted the analysis of the entire known miRNAome. In addition, other methods such as bead-flow cytometry, quantitative real-time polymerase chain reaction, and high-throughput array-based Klenow enzyme assay have been used to assess miRNA expression in tumors and other diseases. To date, altered miRNA expression had been reported in almost all types of cancer [41].

In 2005, the first reports addressing the biological function of miRNAs in cancer were published. MicroRNAs can act as oncogenes (oncomirs) or tumor suppressors and are involved in a variety of pathways deregulated in cancer [42]. In March 2005, Johnson et al. reported the first miR-target interaction with relevance to cancer. The authors demonstrated that in *C. elegans* let-7 targets let-60, encoding the *C. elegans* ortholog of human oncogene RAS. In addition, they showed that human RAS expression is regulated by let-7 in cell culture. Accordingly, let-7 expression is decreased in lung cancer compared with normal tissue, and it correlates with the increased RAS protein levels detected in lung tumor samples [43]. Cimmino et al. reported that miR-15 and miR-16, the first two miRNAs associated with cancer, play a role in apoptosis regulation by targeting the anti-apoptotic BCL2 mRNA [44]. Also in 2005, He et al. studied for the first time the contribution of miRNAs to tumor development *in vivo*. By overexpressing the miR-17-22 cluster, which is upregulated in human lymphoma, they were able to accelerate lymphomagenesis in a mouse B-cell lymphoma model carrying c-myc oncogene [45]. Furthermore, O'Donnell et al. reported for the first time that a transcription factor, specifically MYC, modulated the expression of the same cluster of miRNAs

and consequently the E2F1 expression [46]. From 2005 until the present, hundreds of scientific communications have reported on the role of miRNAs in tumors, as well as the regulation of miRNAs by other transcription factors such as TP53 (for a detailed review see [47]). In 2010, Medina et al. reported results in mice that conditionally overexpressed miR-21 (without other predisposing genetic backgrounds), clearly demonstrating that overexpression of a single miRNA, specifically miR-21, was sufficient to cause tumor development. In addition, these authors proved that tumor volume and survival were dependent on miR-21 overexpression and that the tumors regressed when miR-21 was inactivated, proving for the first time an oncogenic miRNA addiction for tumor cells [48].

MicroRNAs deregulation can be caused by several mechanisms including deletion, amplification, mutation, or dysregulation of transcription factors that target specific miRNAs. In addition, miRNAs can be controlled by epigenetic mechanisms. In 2006, Saito et al. were the first to demonstrate that miRNAs expression could be controlled by the two major epigenetic mechanisms: DNA methylation and histone modifications [49,50]. When Saito et al. simultaneously treated a human bladder carcinoma cell line with 5-aza-2-deoxycytidine (a DNA methylation inhibitor) and 4-phenylbutyric acid (a histone deacetylase inhibitor), 17 of 313 human miRNAs were found to be upregulated. In particular, miR-127 was upregulated after treatment with these drugs and expression of one of its targets, BCL6, was suppressed [49]. Accordingly, miR-127 expression was downregulated in primary human bladder and prostate tumors compared with normal tissue [49]. Therefore, these authors concluded that in cancer tissue miR-127 is subject to epigenetic silencing [49]. This finding opened the field of epigenetics to miRNAs regulation [50]. Interestingly, a bidirectional connection between epigenetics and miRNAs has been established. On the one hand, epigenetic mechanisms control miRNAs; on the other hand, miRNAs can target essential epigenetic key players. One of the first reports suggesting methylation could be controlled by miRNAs was published in 2004; Bao et al. suggested that miR-165/166 are important for methylation of *PHB* gene in *Arabidopsis* [50,51].

In cancer patients, metastasis is the principal cause of death. The metastatic process involves multiple steps: cell motility, invasion of adjacent stroma, intravasation, systemic dissemination (though either the blood or lymph), extravasation into the parenchyma of distant tissues, and finally proliferation at a new site, giving rise to secondary tumor. In this process, miRNAs have a dual role as they can promote or inhibit metastasis [52]. The first finding about miRNAs functioning as metastasis activators was reported by Ma et al. [53]. MiR-10b positively regulates cell migration and invasion *in vitro* and is capable of initiating tumor invasion and metastasis *in vivo*. MiR-10b acts by directly targeting HOXD10, which is a transcriptional repressor of RHOC, a key player in metastasis. Concordantly, miR-10b expression is elevated in about 50% of metastatic breast tumors compared with metastasis-free tumors or normal breast tissues [53]. Along the same line, Huang et al. found that human miR-373 and miR-520c stimulated cancer cell migration and invasion *in vitro* and *in vivo* [54].

In contrast, miRNAs can prevent tumor metastasis. Tavazoie et al. published the initial study of miRNAs as metastasis suppressors [55]. In breast cancer, patients with low expression levels of miR-335, miR-126, and miR-206 had a shorter median time to metastatic relapse. Restoration of their expression in breast cancer cell lines decreased the number of metastases in inoculated mice. These miRNAs have distinct mechanisms for metastasis suppression: restoration of miR-126 expression significantly suppressed overall tumor growth, whereas restoration of miR-335 or miR-206 levels altered cell morphology, possibly causing a decrease in cell motility [55].

It was therefore not surprising that Lujambio et al. reported in 2008 that DNA methylation-associated silencing of tumor-suppressor miRNAs contributed to the development of human cancer metastasis and that the reintroduction of miR-148a and miR-34b/c in cancer cells with epigenetic inactivation inhibited their motility, reduced tumor growth, and inhibited metastasis formation in xenograft models, with an associated downregulation of the miRNA oncogenic target genes, such as C-MYC, E2F3, CDK6, and TGIF2 [56].

5.2. MicroRNAs in cardiovascular diseases

In 2005, Zhao et al. reported that miR-1-1 and miR-1-2 were specifically expressed in cardiac and skeletal muscle precursor cells and that miR-1 regulates ventricular cardiomyocytes. MiR-1 overexpression specifically in the developing heart of a mouse model led to a decreased pool of proliferating ventricular cardiomyocytes [57]. In the same year, Kwon et al. found that miR-1 in *Drosophila* modulates cardiogenesis [58]. Subsequently, Chen et al. in 2006 reported that miRNA-1 promotes myogenesis, whereas miR-133, clustered together with miR-1 on mouse chromosome 2, stimulates myoblast proliferation [59]. These three reports were the first to suggest a possible involvement of miRNAs in cardiac-related human diseases. van Rooij et al. published the first report associating miRNAs with heart failure and cardiac hypertrophy [60], which occurs in response to stress and injury and is characterized by an increase in cardiomyocyte size with no change in myocyte number [61]. By miRNA microarray analysis, more than 12 miRNAs were identified as deregulated during cardiac hypertrophy and heart failure [60]. Interestingly, overexpression of miR-195 led to heart failure in a mouse model and to hypertrophic growth of cultured rat cardiomyocytes [60,61]. Several subsequent studies demonstrated miRNA aberrant patterns in cardiac hypertrophy and analyzed their roles [61], including that of miR-21, which is also one of the most deregulated miRNAs in cancer [62,63], suggesting common miRNA pathways involved in both abnormal states.

5.3. MicroRNAs in autoimmune diseases

Psoriasis is the most prevalent chronic inflammatory skin disease in adults and the first inflammatory disease in which miRNAs were implicated [64]. In 2007, Sonkoly et al. found that miR-203, which is expressed in keratinocytes, is upregulated in psoriasis-affected skin compared with healthy human skin or another chronic inflammatory skin disease. Interestingly, miR-203 targets SOCS-3 [64]. It had previously been found that SOCS-3 suppression leads to sustained activation of STAT3 in response to IL-6 and that constitutive activation of STAT3 in keratinocytes leads to the spontaneous development of psoriasis in mice [64–66]. MiR-146 is the other miRNA that Sonkoly et al. found associated with psoriasis. It was found upregulated in psoriasis patients and is absent from keratinocytes and dermal fibroblasts but expressed by immune cells [64]. The upregulation of a miRNA associated with inflammation is not surprising because results from several studies support a role of a deregulated immune system in psoriasis [67]. MiR-146 targets IRAK1 and TRAF6, both regulators of the TNF-α signaling pathway [68]. Treatments using anti-TNF agents such as infliximab, etanercept, and adalimumab are effective for psoriasis [69,70]. Therefore, future studies should address treatments with miR-203 and miR-146 antagomirs for psoriasis patients to improve their quality of life [64].

Rheumatoid arthritis (RA) is a systemic autoimmune disorder characterized by chronic inflammation of synovial tissue [71]. In 2008, three studies reported the first association studies between RA and miRNAs. Stanczyk et al. found increased expression of miR-155 and miR-146 in RA synovial fibroblasts and RA synovial tissue [72]; one month later, Nakasa et al. confirmed the expression of miRNA-146 in RA synovial tissue and further described its induc-

tion by stimulation with TNF and IL-1 [73]. In the same year, Pauley et al. reported increased expression of miR-146 in RA peripheral blood mononuclear cells, similar to the results found in synovial tissue/fibroblasts [74]. Taken together, these results suggest that miR-146 can be used as a biomarker for RA. In addition, several other miRNAs have been implicated in RA, such as miR-155, miR-132, miR16 [75], miR-346 [76], and miR-223 [77].

For lupus erythematosus (SLE), an autoimmune disorder characterized by excessive production of a variety of auto-antibodies against several self-antigens [78], few studies have been published about the differently expressed miRNAs. Nonetheless, miRNAs might be implicated in the etiology of this disease, and evidence suggests that miRNAs are potential diagnostic markers in SLE [79–82]. Dai et al. described for the first time 16 miRNAs differently expressed in SLE [79]. These authors were also the first to describe 66 miRNAs differently expressed in patients with lupus nephritis [83]. Several studies in 2010 confirmed the link between miRNAs and SLE/lupus nephritis. Two miRNAs (miR-21 and miR-148a) are overexpressed in CD4+ T cells from patients with lupus. MiR-148a directly downregulates DNMT1 expression and miR-21 indirectly downregulates DNMT1 expression by targeting RASGRP1, an important autoimmune gene. By causing DNMT1 downregulation, both miRNAs contribute to DNA hypomethylation in lupus CD4+ T cells [80]. Te et al. analyzed miRNAs in lupus nephritis patients from two racial groups (African American and European American) and concluded that five miRNAs (miR-371-5P, miR-423-5P, miR-638, miR-1224-3P, and miR-663) were differentially expressed across different racial groups [81]. Recently, Zhao et al. showed that miR-125 expression was reduced, and accordingly expression of its predicted target KLF13 was increased, in SLE patients [82].

Although further studies with larger numbers of samples are necessary, these studies implicate miRNAs in immune disease pathogenesis and suggest that they can be used as potential diagnostic markers.

5.4. MicroRNAs in neurodegenerative diseases

A significant number of miRNAs is specifically expressed in the central nervous system and plays a role in neuronal development [84]. Consequently, it seems natural that miRNAs have been linked to neurodegenerative diseases such as Alzheimer's, Parkinson's, and Huntington's disease, which are caused by excessive neuronal death in the diseased brain [85]. In 2007, there were reported the first studies associating miRNAs with neurodegenerative diseases. Using Purkinje cells, Schaefer et al. suggested an involvement of miRNAs in neurodegenerative disorders by showing progressive neurodegeneration (cerebellar degeneration and development of ataxia) in the absence of the miRNA biogenesis key component DICER, which consequently causes progressive loss of miRNAs [86,87]. Lukiw et al. found that miR-9, miR-25b, and miR-128 were upregulated, and that miR-124a was downregulated, in Alzheimer's disease brain (hippocampal region) samples when compared with aged-match controls [88,89]. In the same year (2007), an miRNA important for maturation and function of dopaminergic neurons, miR-133b, was one of the first miRNAs reported to be lost in midbrain tissue in Parkinson's disease [90].

MiRNA expression profiles for neurodegenerative diseases are challenging for two main reasons: (1) some miRNAs are expressed specifically by only some brain regions or brain cell types; and (2) unlike cancer, it is much more challenging to obtain disease-affected and healthy tissue from the same patient, which in turn makes it more difficult to control these types of studies [91]. Since 2007, several studies have linked miRNA modifications to regulation of proteins involved in these diseases [92]. Although studies seem to indicate that miRNAs can participate in neurodegenerative disease initiation or progression, more work is necessary to clarify whether changes in miRNA expression directly contribute to the pathogenesis of neurodegenerative diseases or whether they are secondary events caused by deregulated pathways in these diseases [89]. It is predicted that in the next few years, increased research on miRNAs will lead to novel insight in this field [91].

6. MicroRNAs as molecular biomarkers

In a clinical context, miRNAs can be extremely useful in disease diagnosis and prognosis and in prediction of therapeutic response. In 2004, Takamizawa and coworkers were the first to pinpoint the prognostic value of miRNAs by showing that let-7 expression was reduced in lung cancers and that lung cancer patients with low let-7 expression levels have a significantly shorter survival after potentially curative resection [93]. In 2005, Calin et al. reported the first study showing the diagnostic/prognostic importance of miRNAs at the genome-wide level [94]. These authors found that miRNA expression profiles could be used to distinguish normal B cells from malignant B cells in patients with CLL. In fact, a unique miRNA expression signature is associated with prognostic factors such as ZAP-70 expression (predictor of early disease progression) and mutational status of IgVh. In addition, these authors found nine miRNAs that were differently expressed between patients with a short interval from diagnosis to initial therapy and patients with a significantly longer interval. Furthermore, this study also highlighted the fact that one mechanism of miRNA deregulation is mutation: a germline mutation in the precursor of miR-16-1-miR15a caused low levels of miRNA expression both *in vitro* and *in vivo* [94].

Currently, the clinical utility of miRNAs as diagnostic/prognostic biomarkers has been demonstrated in several types of cancer by numerous studies using tumor samples removed during surgery or biopsies [42]. For non-malignant diseases, however, larger and more studies should be conducted.

7. MicroRNAs as biomarkers in plasma or serum

Current techniques for cancer diagnosis commonly involve a biopsy of the cancer tissue. Because this technique is invasive and unpleasant for patients, some studies have been focused on the search for biomarkers in human fluids such as plasma/serum, urine, or saliva. Blood samples from patients are usually readily available and many biological molecules, as circulating nucleic acids, can be found in blood serum/plasma, including miRNAs [95,96]. These small non-coding RNAs in the blood are incorporated into microparticles and exosomes (50- to 90-nm membrane vesicles) that prevent their degradation, conferring an advantage to the use of miRNAs as markers in serum [95]. In addition, detection of miRNAs in serum is easy owing to highly sensitive PCR detection methods, the lack of post-processing modifications of miRNAs, and simple methods of miRNAs extraction from serum [95]. The first report addressing the utility of miRNAs as diagnostic tools in biological fluids was published in 2008 by Chim et al. in a study that detected placental miRNAs in the maternal plasma [97]. In the same year, Lawrie et al. [98], by comparing serum from patients with diffuse large B-cell lymphoma and healthy controls, found that miR-155, miR-210, and miR-21 levels were significantly upregulated in patients. Interestingly, these miRNAs have been shown to be deregulated in tumors. Moreover, high expression of miR-21 in patients serum was correlated with improved relapse-free survival times [98]. To date, miRNAs deregulation in serum of cancer patients have been described for several cancers, including leukemia, lymphoma, and gastric, colorectal, lung, oral and squamous cell, breast, ovarian, prostate, pancreatic, and hepatocellular cancers [96].

Furthermore, a recent report analyzed miRNAs plasma levels in patients with RA or osteoarthritis. Remarkably, plasma miR-132 was significantly higher in healthy controls than in RA or osteoarthritis patients and therefore it can be a potential diagnostic marker. In addition, plasma miRNA levels correlated with disease activity in RA. However, miRNAs included in this study failed to differentiate between RA and osteoarthritis. Thus, other miRNAs specific to each disease should be studied. Interestingly, this study also analyzed miRNAs present in synovial fluid and demonstrated that miRNAs from plasma and from synovial fluid have different origins [99,100].

To translate the evaluation of miRNAs expression in serum into a clinical routine for diagnosis and prognosis, it is still necessary to standardize the methodologies used for these studies, i.e., serum/plasma extraction procedures and storage conditions, housekeeping miRNAs for normalization in serum samples, or the use of the same statistical methods for data analysis [95]. In addition, large studies reporting miRNA levels in plasma and serum with detailed clinical data information, together with normal controls from both sexes and different ages, are still needed [96].

8. Therapeutic implications

MiRNAs are aberrantly expressed in several diseases; therefore, it is not surprising that these small ncRNAs represent potential therapeutic targets for the diseases they are functionally associated with. MiRNAs that are upregulated in diseases should be targeted using anti-miRNAs, which are antisense oligonucleotides with specific modifications [101]. For instance, antagomirs, a class of anti-miRNAs that is cholesterol-conjugated to facilitate cellular intake and serum protein binding, could be used to block oncomirs in cancer [101,102]. In 2004, Hutvágner et al. successfully copied the phenotype of let-7 loss-of-function mutation by injecting a 2′-*O*-methyl oligonucleotide complementary to let-7 miRNA into *C. elegans* larvae [103]. In 2005, Krützfeldt et al. reported for the first time the use of antagomirs *in vivo* in mammals [104]. Using a mouse model, Krützfeldt and colleagues systemically delivered via intravenous injection antagomirs against miR-16, miR-122, miR-192, and miR-194 that specifically downregulated the corresponding miRNAs. Silencing of miRNAs using antagomirs was long lasting, and miR-16-antagomir effects were detected in multiple tissues, except in the brain, possibly due to the blood-brain barrier [101,102,104,105]. Other approaches to efficiently inhibiting miRNAs *in vivo* include the use of locked nucleic acid (LNA) oligos or 2′-*O*-methoxyethyl phosphorothioate (MOE) modification [102]. Elmén et al. evaluated for the first time the effect of an LNA-anti-miRNA in non-human primates, with surprising results. These authors intravenously injected an LNA-anti-miRNA-122 into African green moneys and were able to efficiently silence the mature miR-122. The effect was long-lasting and safe, as neither toxicity associated with LNA nor histopathological changes were detected [106]. Two years later, Lanford et al. described the utility of anti-microRNAs for the clinical practice. MiR-122 is a liver-expressed miRNA essential for hepatitis C virus (HCV) replication. Using an LNA-anti-miR-122, the authors were able to suppress HCV viremia in chronically HCV-infected chimpanzees. Moreover, this therapy generated a high barrier to resistance, and no side effects were detected [107].

In addition to these direct-inhibitory methodologies, an indirect technology can be used through downregulation of miRNA biogenesis pathway components. Tetracycline-inducible shRNAs could be used to downregulate Dicer or Drosha, key components of the miRNA-biogenesis pathway; however, this mechanism should be tightly controlled, as downregulation of this pathway will have an effect on all miRNAs [101,102].

On the other hand, when miRNAs downregulation promotes disease, as it is the case of some miRNAs downregulated in tumors and that function as tumor suppressors, a therapeutic approach would be to restore the mature miRNA levels in the proper tissue/cells. In this situation, it should be used synthetic RNA duplexes, resembling siRNA molecules, that will mimics miRNAs duplex and will be recognized by RISC complex [102]. This complex will process it as the endogenous miRNA, by loading the stand with the sequence identical to the mature miRNA. This approach still needs to be evaluated *in vivo* because some challenges, such as stability and delivery strategies, need to be improved [101,102,108]. The use of short-hairpins driven by Pol III promoters could be a strategy for re-expression of miRNAs in the cells [108], although the dosage used in this mechanism has to be narrowly controlled, since sustained shRNA high-level expression in livers of adult mice has been shown to be fatal [109]. In 2009, Kota et al. demonstrated how useful systemic administration of miRNAs can be for anti-cancer therapy. MiR-26a is expressed at low levels in hepatocellular carcinoma but normally expressed in other tissues. These authors used an adeno-associated virus to mediate miR-26a delivery in a mouse model of liver cancer and were able to reduce cancer cell proliferation and induce tumor cell apoptosis, which consequently caused tumor regression. Since only cancer cells present miR-26a downregulation, the delivery was highly specific and did not affect normal tissue, which was tolerant to miR-26a restoration [110].

9. Concluding remarks

MiRNAs were discovered in 1993 and rapidly became an exciting topic of research during the last decade, with the number of published studies growing exponentially. Like miRNAs, other ncRNAs have recently been linked to diseases [111,112]. Therefore, we may predict that our understanding of how other classes of ncRNAs mediate disease regulation will advance rapidly. Also, we fully acknowledge that before the "miRNA revolution" started, the literature was recording few pioneering studies on the roles of long RNAs that do not codify for proteins (for review see Mattick [113]). It is expected that in future years the acquired biological knowledge concerning miRNAs can be widely translated into the clinic. Despite major challenges that still need to be overcome (such as tissue-specific delivery), miRNAs hold great potential as therapeutic targets.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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MicroRNA Down-Regulated in Human Cholangiocarcinoma Control Cell Cycle Through Multiple Targets Involved in the G1/S Checkpoint

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MicroRNAs (miRs) recently emerged as prominent regulators of cancer processes. In the current study we aimed at elucidating regulatory pathways and mechanisms through which miR-494, one of the miR species found to be down-regulated in cholangiocarcinoma (CCA), participates in cancer homeostasis. miR-494 was identified as down-regulated in CCA based on miR arrays. Its expression was verified with quantitative real-time reverse-transcription polymerase chain reaction (qRT-PCR). To enforce miR expression, we employed both transfection methods, as well as a retroviral construct to stably overexpress miR-494. Up-regulation of miR-494 in cancer cells decreased growth, consistent with a functional role. mRNA arrays of cells treated with miR-494, followed by pathway analysis, suggested that miR-494 impacts cell cycle regulation. Cell cycle analyses demonstrated that miR-494 induces a significant G1/S checkpoint reinforcement. Further analyses demonstrated that miR-494 down-regulates multiple molecules involved in this transition checkpoint. Luciferase reporter assays demonstrated a direct interaction between miR-494 and the 3′-untranslated region of cyclin-dependent kinase 6 (CDK6). Last, xenograft experiments demonstrated that miR-494 induces a significant cancer growth retardation *in vivo*. *Conclusion:* Our findings demonstrate that miR-494 is down-regulated in CCA and that its up-regulation induces cancer cell growth retardation through multiple targets involved in the G1-S transition. These findings support the paradigm that miRs are salient cellular signaling pathway modulators, and thus represent attractive therapeutic targets. miR-494 emerges as an important regulator of CCA growth and its further study may lead to the development of novel therapeutics. (HEPATOLOGY 2011;54:2089-2098)

Cholangiocarcinomas (CCAs) are epithelial cancers of the biliary tree.[1] CCAs are usually diagnosed late in their progression and patient survival is usually measured in months.[2] Primary sclerosing cholangitis (PSC) is a major CCA risk factor in the U.S., whereas infection with *Opistorchis viverrini* and *Chlonorchis sinensis* represents a major CCA risk factor in Southeast Asia.[3,4] These observations lead to the hypothesis that inflammation in the biliary tree is a major predisposing factor to cancer formation.

Abbreviations: APC, allophycocianin; BrdU, bromodeoxyuridine; CCA, cholangiocarcinoma; CCND1, cyclin D1; CCNE2, cyclin E2; CDK4, cyclin-dependent kinase 4; CDK6, cyclin-dependent kinase 6; DMEM, Dulbecco's Modified Eagle's Medium; eGFP, enhanced green fluorescence protein; FCS, fetal calf serum; HDAC1, histone deacetylase 1; HuCCT1-EV, HuCCT1-MIEG3-empty control; HuCCT1-494V, HuCCT1-MIEG3-miR494; IPA, ingenuity pathway analysis; IRES2, internal ribosome entry site 2; MIEG3, MSCV-IRES-enhanced-GFP-3; miR, microRNA; NBD, normal biliary duct epithelia; NSM, nonspecific mimic; P/S, penicillin/streptomycin; PSC, primary sclerosing cholangitis; qRT-PCR, quantitative real time RT-PCR; UTR, untranslated region.

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Molecular characterization of CCAs[5] further suggested that inflammation and cholestasis, through modulation of genes involved in DNA damage repair, promote cancer development.

MicroRNAs (miRs) are short, single-stranded sequences of RNA that were recently demonstrated to play a major role in the regulation of virtually all cellular processes.[6,7] In addition, microRNAs were also implicated in all solid cancers evaluated to date.[6,8,9] miRNAs act mainly by decreasing protein expression at a posttranscriptional level, largely through nucleotide complementarity to the 3′ untranslated region (UTR) of corresponding species of messenger RNA (mRNA).[10]

The involvement of miRs in the genesis or homeostasis of CCA was reported in several studies. Alterations of miR expression was first reported in CCA cell lines,[11] then in human tissues.[12] Subsequent studies demonstrated that the expression of miRs-7a, -29, and -370 is linked to cholangiocarcinogenesis, either through an interleukin (IL)-6-dependent pathway, or by interacting with Mcl-1.[13-16] Further work linked miRs to cholangiocyte immune responses to infection, suggesting miR implication in inflammation-derived carcinogenesis.[17-20]

One major hurdle in identifying miR roles and mechanisms in cancer results from the high number of predicted targets for any single miR species.[21] Nonetheless, experimental validation confirms only a small fraction of these targets.[21] To complicate matters, conserved miR binding sites are as widespread in the open reading frame as they are in the 3′UTR, and are also common in the 5′UTR regions.[22] Therefore, employing *in silico* search engines as a sole modality to identify biologically relevant targets appears to have relatively low accuracy. Fortunately, recent work demonstrated that decreasing amount of the target mRNA species account for ≈84% of the miR effects on protein expression.[23] Therefore, it appears that screening for alterations in mRNA levels in response to miR manipulation through either mRNA arrays or sequencing offers a valuable complement to search strategies employing *in silico* engines.

In the current study we found that miR-494 is down-regulated in human CCAs. To obtain a comprehensive and unbiased view regarding the effects of miR-494 in cancer cells, we performed mRNA arrays on cells overexpressing miR-494 and on negative control. By employing pathway analysis and then confirming the results with western blotting we found that miR-494 exerts moderate effects on multiple molecules along the canonical G1-S transition pathway. These actions appear to converge to restore the G1-S checkpoint, which explains, at least in part, the delayed growth of cells expressing miR-494.

Materials and Methods

Human Tissues. The human specimens were obtained at surgery performed at the Johns Hopkins Hospital, the Mayo Clinic, and Fundeni Clinical Institute. The normal bile duct (NBD) specimens were obtained from surgical resections performed for other cancers. Informed consent was obtained from all patients.

Cell Lines. HuCCT1 and TFK1 cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal calf serum (FCS), 1000 U/mL penicillin/streptomycin (P/S), as described.[24] H69 cells, a gift from Dr. D. Jefferson (Tufts University, Boston, MA), are normal human intrahepatic cholangiocytes transformed with SV-40. They were derived from a normal liver prior to liver transplantation.[25]

RNA Extraction. Total RNA extraction was performed by lysing cells in TRIzol reagent (Invitrogen, Carlsbad, CA).

Quantitative Real-Time Reverse-Transcription Polymerase Chain Reaction (qRT-PCR) for miR Expression. We performed miR qRT-PCR to evaluate the expression of candidate miRs. TaqMan miR Assays (Applied Biosystems, Foster City, CA) were used. Cycle passing threshold (Ct) was recorded and normalized to RNU6B expression. Relative expression was calculated as $2^{Ct\_miR\text{-}Ct\_RNU6B}$. PCR reactions were carried out in duplicate.

Transfection of miR Mimic. The synthesized miR-494 mimic was purchased from Dharmacon (Lafayette, CO). Approximately 30%-50% confluent cells were

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transfected with 60 nM of miR-494 mimic or mimic-negative control using Lipofectamine RNAi MAX (Invitrogen). RNA and proteins were harvested 72 hours after transfection.

Western Blotting. Western blotting was performed per standard protocols. Antibodies to Phospho-Rb, CDK4, CCND1, and CCNE2 were purchased from Cell Signaling, and CDK6 was purchased from Santa Cruz Biotechnology.

Cell Counting. Ten thousand cells were plated in 24-well plates (day 0), transfected 24 hours later (day 1), and counted daily for a total of 5 days (days 2-6) using a hemocytometer and an inverted-light microscope.

Complementary DNA (cDNA) Picroarrays and Filtering Genes. The Illumina cDNA microarray platform in the Johns Hopkins genomics facility was used for cDNA microarrays. Cells were treated with miR-494 or NSM and 72 hours later the RNA was extracted. Candidate genes were filtered as follows: genes with expression in either HuCCT1 or TFK1 cells under 3,000 units were eliminated from analysis due to low expression. Genes that demonstrated less than a 20% decrease in both HuCCT1 and TFK1 cells upon stimulation with miR-494 were eliminated. From 24,527 tags, the list of genes was reduced to 137. These genes were input into ingenuity pathway analysis (IPA) to identify the pathways in which they are involved.

Proliferation Assay with Bromodeoxyuridine (BrdU) Incorporation. At 72 hours posttransfection, cells were cultured for 10 minutes with 10 μM BrdU in DMEM. Subsequently, the cells were fixed and permeabilized, then treated with 100 μL PBS, 300 μg/mL DNase I (BD PharMingen) for 1 hour at 37°C in the dark. After washing, the cells were stained with allophycocianin (APC)-labeled anti-BrdU antibody for 20 minutes at room temperature in the dark and analyzed by FACSCalibur.

Cell Cycle Analysis by Flow Cytometry. Cells were incubated with propidium iodide (PI) staining buffer (phosphate-buffered saline [PBS] 0.1 mg/mL PI, 0.6% NP40, 2 mg/mL RNase A for 30 minutes on ice [Roche Diagnostics]). The DNA content was analyzed using FACSCalibur (BD Biosciences, San Jose, CA) and Cell Quest software (BD Biosciences). Nocodazole treatment, where applicable, was performed 24 hours prior to harvesting cells at a final concentration of 100 ng/mL.

Retroviral Vectors, Viral Supernatant Production, and Viral Transduction. MSCV-based bicistronic retroviral vector, MIEG3[26] was used to express miR-494. The genomic DNA sequence from −80 to +80 of miR-494 was amplified using PCR primers flanked by EcoRI (5′) and XhoI (3′) and cloned into the multiple cloning site of MIEG3. The expression of miR-494 was linked with expression of enhanced green fluorescence protein (eGFP) by way of internal ribosome entry site 2 (IRES2).

The plasmid DNA was used to generate viral supernatant from Phoenix-gp cells as described.[27] To stably express miR-494, 1×10^5 HuCCT cells were incubated with 3 mL of viral supernatant containing 8 mg/mL of hexadimethrine bromide (Polybrene, Sigma-Aldrich, Milwaukee, WI). After 6-8 hours, the viral supernatant was discarded and fresh DMEM was added. Two days after transduction, cells were harvested and sorted for eGFP expression.

Luciferase Reporter Assay. A portion of the CDK6 3′UTR, containing miR-494 predicted binding site, was amplified using linker primers containing *Xba*I restriction sites. Next we employed the Gene Tailor Site-Directed Mutagenesis System (Invitrogen) to introduce mutations in the miR-494 binding site. The sequences of primers is provided in the Supporting Materials. After sequence verification, 6,000 cells per well were seeded onto 96-well plates on the day prior to transfection. Cells were transfected with miR-494 mimic (Dharmacon) or the control, then with the pGL3 vector and an internal control pRL-CMV (Renilla luciferase). Forty-eight hours later the luciferase reporter assay was performed using a Dual-Glo Luciferase Assay System (Promega). The luminescence intensity of firefly luciferase was normalized to that of Renilla luciferase. The effect of miR-494 on the wild-type or mutant CDK6 3′UTR was calculated as a fraction of the effect exerted by the negative control (NSM). The raw data allowing direct comparison of wildtype and mutant CDK6 3′UTR luciferase activities are available in Supporting Materials.

Subcutaneous Tumor Formation. HuCCT1-MIEG3-E and HuCCT1-MIEG3-miR494 were grown in T175 plastic flasks. Then 3.25 million cells were resuspended in 350 μL of PBS and 200 μL of Matrigel. The cells were injected subcutaneously into 6-week-old NOD/SCID mice (strain 394) purchased from Charles River Laboratories. Tumors were measured with a caliper. The reason we chose the NOD/SCID mouse model instead of the SCID mouse was based on the following rationale: Severe combined immune-deficient (SCID) mice have T and B cell deficiencies[28]; however, they retain residual immunity from natural killer (NK) cells and complement. The nonobese diabetic (NOD)/SCID mice represent a

Table 1. miR-494 Is Overexpressed in Human CCA Versus Normal Biliary Epithelium

	Mean NBD	Mean CCA	NBD/CCA	*t*-test
hsa-miR-494	300.37	32.25	9.314657911	0.01
hsa-miR-370	137.87	13.82	9.973922306	0.00
hsa-miR-513	46.04	3.70	12.45113096	0.00
hsa-miR-188	31.37	2.05	15.28287383	0.00
hsa-miR-560	8.53	1.17	7.298486017	0.02

Microarray data is presented. The top five downregulated miRs in CCA are displayed. The miRs are ordered based on the expression of miR-494 in normal biliary epithelium. NBD, normal biliary duct epithelium; CCA, cholangiocarcinoma; NBD/CCA, average expression in NBD divided by average expression in CCA; *t*-test, unpaired Student's *t*-test.

theoretical advantage for tumor xenotransplantation compared to the SCID mice because they have less residual immunity resulting from defects in complement pathway and macrophage function.[29] In addition, NOD/SCID mice housed in clean conditions may have fewer NK cells.[29] Due to these considerations, we thought that choosing NOD/SCID mice for our xenotransplantation experiments might offer higher tumor engraftment.

Results

We previously identified miR species dysregulated in CCA.[28] For the purpose of this study we concentrated on miRs that were down-regulated in CCA versus normal biliary duct epithelia (NBD). The arrays were performed on five NBDs and five CCAs. Data were filtered as described.[30] In brief, raw expression data less than five were considered to be at background levels. We then performed 75th percentile normalization. miR species demonstrating a statistically significant difference (unpaired Student's *t* test) between the CCA and NBD group were retained for further analyses. The candidate miRs were then ordered by the mean expression in NBDs, because a higher expression in normal tissue is suggestive of a putative role, which might be lost in cancer. The top five miRs are displayed in Table 1. We selected miR-494 for all subsequent studies. To confirm these initial miR array data, real-time qRT-PCR analysis was performed using 12 human CCA as well as five normal cholangiocyte specimens. As shown in Fig. 1A, miR-494 is uniformly and significantly down-regulated in human CCA specimens versus normal specimens. In addition, the level of miR-494 in a transformed normal cholangiocyte cell line, H69, was similar to the level found in NBDs. Correspondingly, the level of miR-494 in a cholangiocarcinoma cell line, HuCCT1, was similar to the level found in human primary CCA specimens (Fig. 1A, last lane). Because H69 and HuCCT1 cell lines closely mimic the levels of miR-494 in normal and malignant cholangiocytes, respectively, they were used as an *in vitro* model to further characterize the function of miR-494 in CCA.

To verify our findings we measured the levels of miR-494 in a larger cohort of CCA specimens that was obtained after the screening experiments. As Fig. 1B demonstrates, miR-494 was found to be statistically significantly down-regulated in a group of 43 CCA versus 30 normal tissues.

To characterize the function of miR-494 in cancer, miR-494-mimic or nonspecific mimic (NSM) were transfected into HuCCT1 cells. Cells transfected with miR-494 showed a significant decrease in growth as early as day 2 and this difference became more obvious at later timepoints (Fig. 2A). Once we established that miR-494 promotes decreased cancer cell growth, we

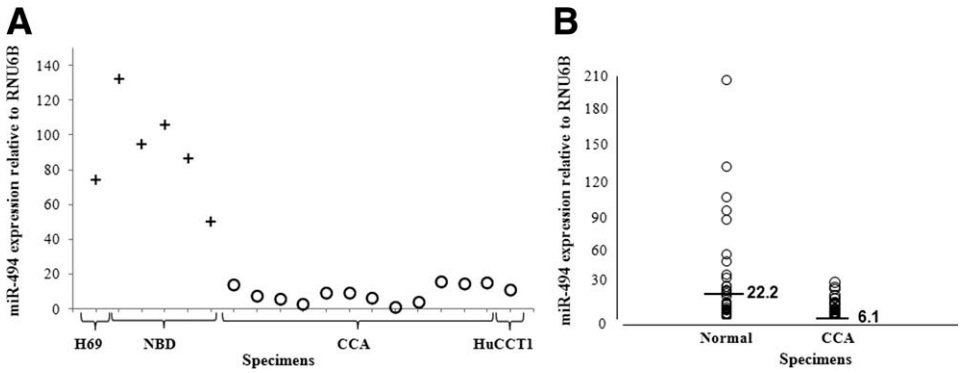

Fig. 1. (A) miR-494 is down-regulated in CCA versus normal biliary epithelium. Crosses, normal cholangiocytes; Circles, malignant cholangiocytes; X-axis, specimens; lane 1, H69 normal cholangiocytes; lanes 2-6, primary human normal biliary epithelium; lanes 7-13, primary human CCA; lane 14, HuCCT1 malignant cholangiocytes. Y-axis, qRT-PCR expression of miR-494 versus RNU6B. (B) qRT-PCR expression of miR-494 is decreased in a large cohort of CCA versus normal. X-axis, normal and CCA specimens, respectively, are shown. Y-axis, expression of miR-494 versus RNU6B.

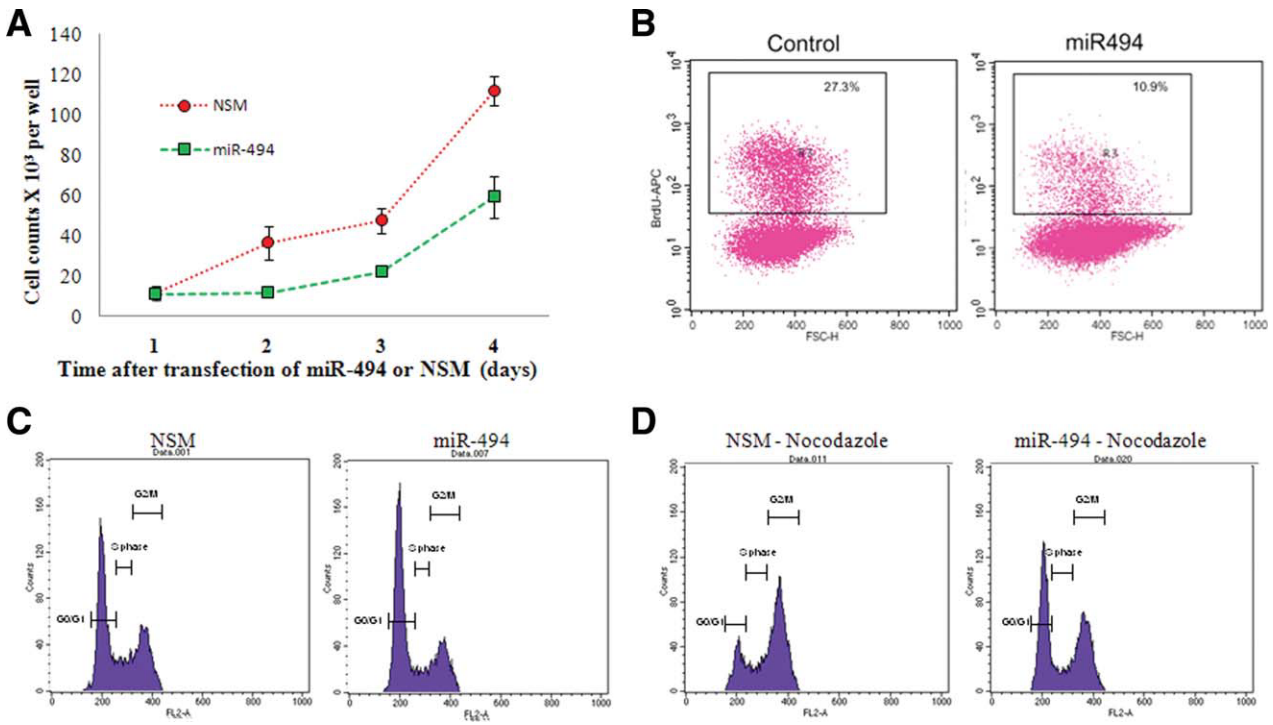

Fig. 2. (A) HuCCT1 malignant cholangiocytes display decreased growth upon miR-494 reinforced expression. X-axis, HuCCT1 cells counted at days 1, 2, 3, and 4 after transfection of miR-494. Y-axis, counts $\times 10^3$ of HuCCT1 cells transfected with miR-494 (green line) or the control NSM (red line). Average of five experiments, n = 5. (B) HuCCT1 malignant cholangiocytes display decreased proliferation upon miR-494 reinforced expression. Flow cytometric analysis of BrdU incorporation of HuCCT1 cells transfected with miR-494 (right panel) or NSM (left panel). Percentage displayed represent percentage BrdU-positive cells of total cells. X-axis, forward scatter (FSC); Y axis, BrdU incorporation, representative of three experiments with three replicates per experiment. (C) HuCCT1 display G1 arrest upon reinforced miR-494 expression. Flow cytometric analysis of cell cycle by way of PI staining of HuCCT1 cells transfected with miR-494 (right panel) or NSM (left panel). X-axis, DNA content as measured by PI incorporation. Y-axis, cell counts for each phase of the cell cycle. This experiment was performed without nocodazole. The figure is representative of three experiments with three replicates per experiment. (D) HuCCT1 cells treated with nocodazole display a more pronounced G1 arrest upon reinforced miR-494 expression. Flow cytometric analysis of cell cycle upon nocodazole treatment of HuCCT1 cells transfected with miR-494 (right panel) or NSM (left panel). X-axis, DNA content as measured by PI incorporation. Y-axis, cell counts for each phase of the cell cycle. This experiment was performed with nocodazole. The figure is representative of three experiments with three replicates per experiment.

sought to further delineate the specific mechanisms underlying its function. First, we confirmed the effects of miR-494 on cancer cell proliferation. Malignant HuCCT1 cells were transfected with miR-494 or NSM and analyzed for BrdU incorporation. As shown in Fig. 2B, HuCCT1 cells transfected with miR-494 have significantly decreased BrdU uptake when compared to cells transfected with NSM, which explains, at least in part, the difference in their growth.

To obtain a mechanistic view into the effects of miR-494 in cancer cells, and because miR-induced destabilization of mRNA is the main reason for decreased protein levels,[23] we stimulated two different CCA cell lines, HuCCT1 and TFK1 cells, with a miR-494 mimic and performed cDNA microarray analysis to quantify changes in mRNA levels. We added the second CCA cell line to circumvent any potential cell line-specific biases. The list of genes identified to be down-regulated upon miR-494 stimulation in both cell lines was then filtered and input into IPA (Ingenuity Systems, Redwood City, CA), with the purpose of identifying general mechanisms of miR function. Of note, this analysis was performed on mRNA species that are reported to be down-regulated by miR-494 on the cDNA arrays, irrespective of presence of binding site in the 3′UTR or *in silico* search engine prediction. IPA reported that the top two networks associated with the list of genes regulated by miR-494 were "Cell Cycle, Antigen Presentation, Cellular Function," and "Cell Cycle, Cancer, Genetic Disorder," respectively (Supporting Table 1). Furthermore, the top-ranked molecular and cellular function of the genes regulated by miR-494 was reported to be "Cell Cycle" (Supporting Table 1).

Data obtained from (1) cell growth and proliferation and (2) cDNA arrays coupled with IPA analysis suggested that miR-494 exerts its function mainly through regulation of cell cycle. To identify the precise effects of miR-494 on cell cycle, we performed cell-cycle analysis by PI staining. These experiments

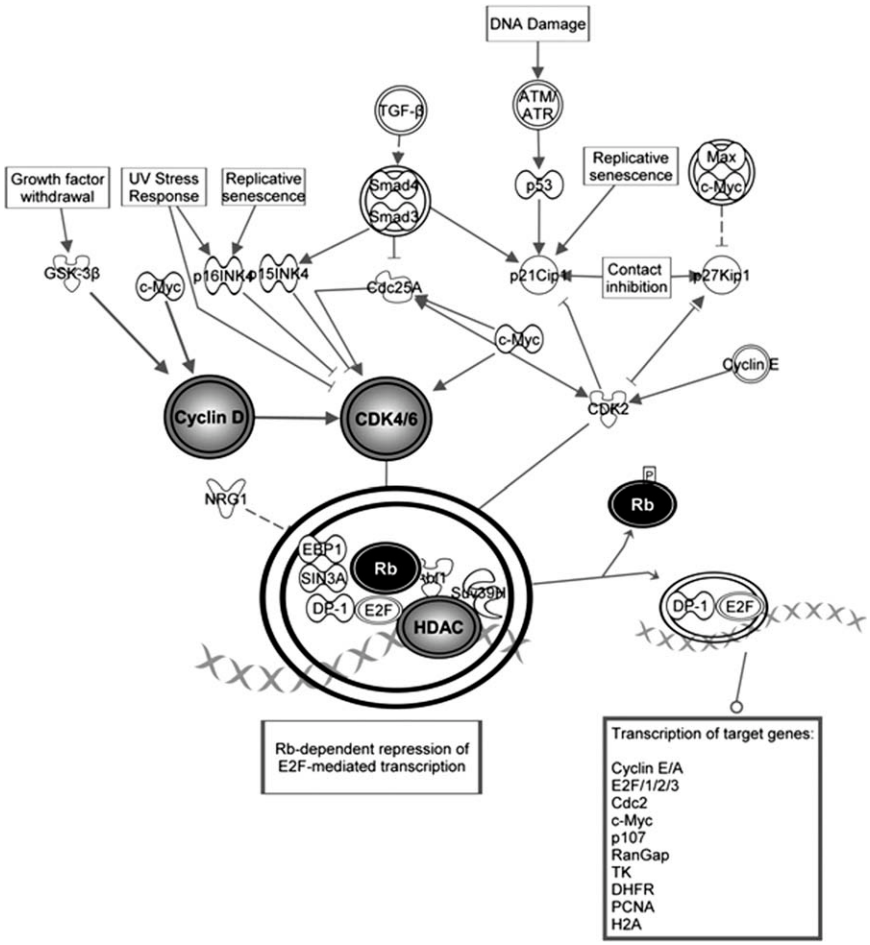

Fig. 3. Genes with altered expression upon miR-494 stimulation are involved in the G1/S checkpoint. CCND1, CCNE2, CDK4, and HDAC1 were identified to be regulated by miR-494. They are involved in the final steps of G1/S checkpoint regulation. The decreasing levels of these molecules results in decreased phosphorylated Rb, with the end result of reinforcement of G1/S checkpoint.

demonstrate an increased in G0/G1 fraction in miR-494 transfected cells (Fig. 2C), consistent with data obtained from BrdU incorporation experiments. This difference becomes more robust upon treatment with Nocodazole, a microtubule-destabilizing agent (Fig. 2D). Interestingly, the down-regulation of miR-494 in HuCCT1 cells has no impact on cell cycle distribution, presumably because of low baseline levels of miR-494 in these cells (Supporting Fig. 1). Of note, the transfection of H69 normal cholangiocytes with miR-494 had no effect on cell cycle progression despite up-regulation of miR-494 by 18-fold (Supporting Fig. 2).

To study the molecular mechanisms responsible for the miR-494-induced G1/S arrest, we queried IPA with regard to genes impacted by miR-494 that are also involved in the G1/S checkpoint. As Fig. 3 shows, based on cDNA microarray data, miR-494 appears to regulate several molecules involved in the G1/S checkpoint. The mRNA levels of cyclin-dependent kinase 4 (CDK4), cyclin-dependent kinase 6 (CDK6), cyclin-D1 (CCND1), cyclin-E2 (CCNE2), and histone-deacetylase-1 (HDAC1) decreased following miR-494 stimulation. We then verified if miR-494 impacts the protein levels of these targets by treating HuCCT1 cells with miR-494 mimic and performing western blotting for these putative targets. As seen in Fig. 4A, expression of miR-494 results in decreased protein levels of CDK6, CDK4, CCND1, CCNE2, and HDAC1. If the effects of miR-494 on these proteins are significant, then, we hypothesized, the final step in the G1 to S transition checkpoint should be affected. We therefore determined whether cells treated with miR-494 showed decreased phosphorylation of Rb. In accord with our hypothesis, we found a decreasing level of phospho-Rb in cells treated with miR-494 (Fig. 4A). We therefore concluded that treatment of cancer cells with miR-494 reinstates the G1/S checkpoint through the coordinated down-regulation of CDK6, CDK4, CCND1, CCNE2, and HDAC1, resulting in decreased phosphorylation of Rb and, finally, delayed cell cycle progression.

The therapeutic up-regulation of miR-494 specifically in cancer cells, without affecting normal surrounding cells, may prove difficult from a practical perspective, in particular if high levels of miR-494

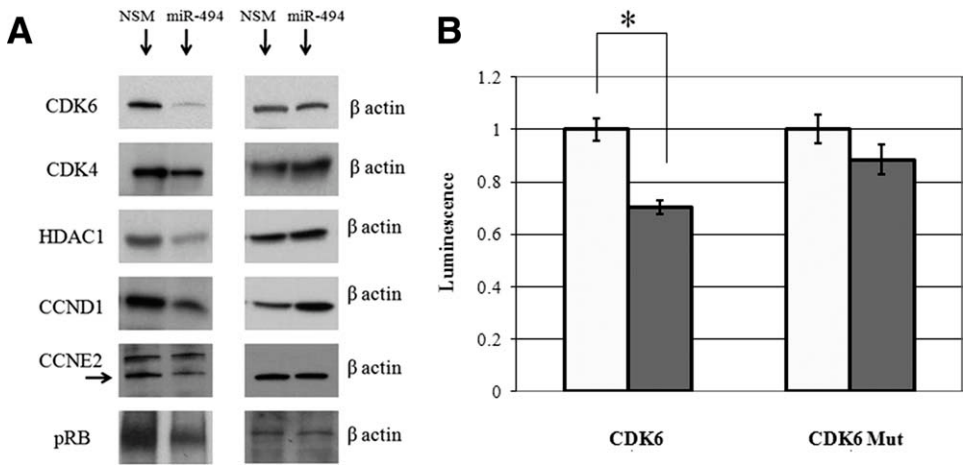

Fig. 4. (A) Protein expression of miR-494 target genes decrease upon miR-494 stimulation. Representative western blots for CCND1, CCNE2, CDK4, HDAC1, and phospho-Rb are shown. Equal protein loading was performed, as shown by β-actin. (B) miR-494 directly interacts with binding site in the 3′UTR of CDK6. Y-axis, relative luminescence normalized to the luminescence level in NSM treatment. X-axis, treatment conditions. NSM, non-specific mimic; 494M, miR-494 mimic; CDK6, correct orientation fragment of CDK6 3′UTR containing miR-494 binding site; CDK6 Mut, fragment of CDK6 3′UTR containing a mutated miR-494 binding site. Shown is the standard error of the mean. miR-494 induces a statistically significant decrease in luminescence ($P < 0.001$, Student's t test) of the forward CDK6 3′UTR fragment versus NSM.

need to be delivered. To accomplish a lower, more physiologic level of miR-494 up-regulation than in transfection experiments, we inserted the genomic locus of miR-494 in a retrovirus, MSCV-IRES-Enhanced-GFP-3 (MIEG3). We then infected HuCCT1 cells with MIEG3-miR-494 and determined the level of miR-494 up-regulation. Compared to cells infected with MIEG3 alone, cells infected with MIEG3-miR-494 displayed a 2.5-fold up-regulation of miR-494 (Supporting Fig. 3A). Of note, this level of miR-494 is higher than in CCA, and close to the level of miR-494 in normal cholangiocytes. In spite of this modest up-regulation of miR-494, cells infected with MIEG3-miR494 behaved similarly to cells transfected with miR-494, displaying restoration of the G1-S checkpoint (Supporting Fig. 3B).

To study the mechanism of miR-494-directed down-regulation of CDK6, CDK4, CCND1, CCNE2, and HDAC1, we searched for conserved binding sites in the 3′UTR of these genes by employing TargetScan (www.targetscan.org). We found that CDK6 is the only gene that has a conserved binding site in its 3′UTR. The binding site is located at position 228-234 in the 3′UTR. A fragment of CDK6 3′UTR, containing the putative miR-494 binding site was cloned into a luciferase vector. We chose to clone a fragment of CDK6 3′UTR because the whole length of the 3′UTR is 10,208 nucleotides. Although cloning the whole 3′UTR would have been ideal, previous experience shows that cloning of large fragments is difficult to achieve due to the adverse effect of size of insert on ligation and transformation efficiencies.[31] In addition, the standard in the field is to clone a fragment of the 3′UTR containing the putative miR binding side into the reporter vector.[32] Cells transfected with the CDK6 3′UTR fragment showed on average a 30% reduction in luciferase activity upon treatment with miR-494 compared to a nonspecific miRNA mimic (NSM). This decrease was statistically significant, with a P-value of less than 0.001 (Student's unpaired t test). Upon miR-494 binding site mutation, the effect of miR-494 on CDK6 was lost, as evinced by similar luciferase activity between miR-494 and NSM-treated cells ($P = 0.15$, Student's unpaired t test, Fig. 4B, Supporting Fig. 4).

To study the effects of miR-494 up-regulation *in vivo*, we injected HuCCT1-MIEG3-empty (control, HuCCT1-EV) and HuCCT1-MIEG3-miR494 (HuCCT1-494V) cells subcutaneously in NOD/SCID mice. Each mouse was injected with 3.25 million HuCCT1-EV cells in the right flank and with 3.25 million HuCCT1-494V in the left flank. Mice formed large tumors in the right flank (HuCCT1-EV), whereas they had very small nodules in the left flank (HuCCT1-494V, Fig. 5A,B). The mice were sacrificed and the tumors removed and analyzed histologically. All three HuCCT1-EV tumors were large and formed almost exclusively of cancer cells. One HuCCT1-494V mass was completely devoid of cancer cells, one HuCCT1-cells was composed mainly of inflammatory cells with no clear evidence of cancer, whereas the third HuCCT1-494V mass had inflammatory cells and very few cancer cells (Fig. 5C; the left panel shows the histology for HuCCT1-EV and the right panel shows the histology for HuCCT1-494V).

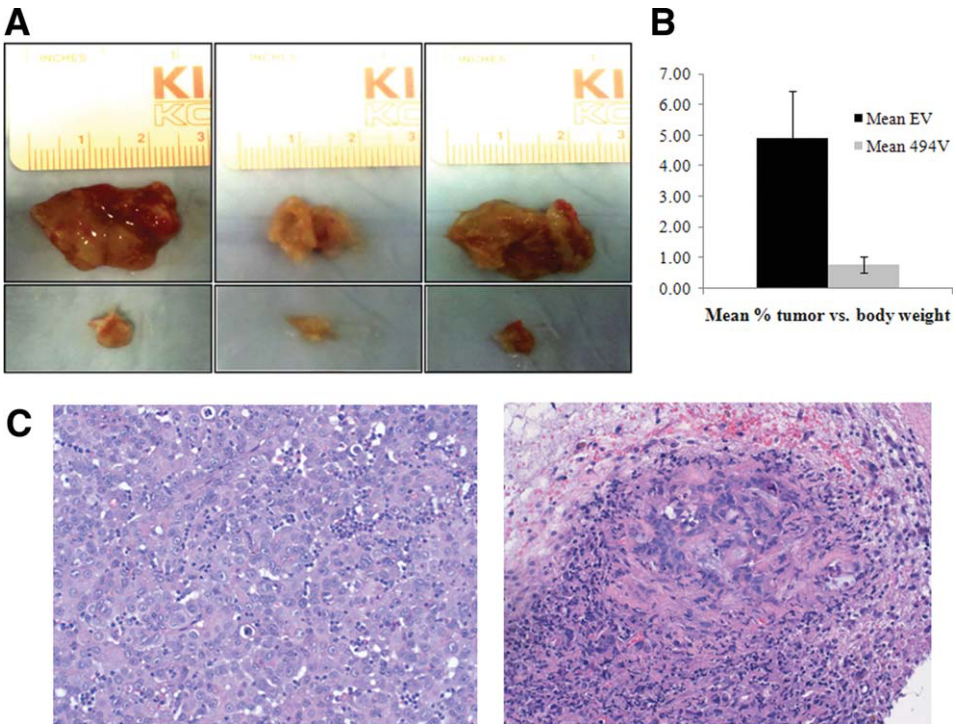

Fig. 5. (A) miR-494 induces cancer growth retardation *in vivo*. HuCCT1-EV and HuCCT-494V were injected in mice. The upper panel shows the tumors formed from HuCCT1-EV cells, which were significantly larger than the tumors formed from HuCCT1-494V cells (lower panel). (B) HuCCT1-494V tumors were significantly smaller than HuCCT1-EV tumors. The mean percent tumor versus mouse body weight are displayed in the figure. (C) HuCCT1-494V masses had no to very few cancer cells, whereas the HuCCT1-EV masses were composed almost exclusively of cancer cells. The HuCCT1-EV tumors were large and composed almost entirely of viable cancer cells. Of the HuCCT1-494V masses, one had no cancer cells, the second showed no clear evidence of cancer cells, and the third (shown in the figure) demonstrates very rare cancer cells amid inflammatory cells and fibrosis.

Discussion

MicroRNAs recently emerged as salient regulators of cancer homeostasis.[33] In addition, there is evidence that miRs may be valuable as *in vivo* therapeutics.[34,35] In the current study, we report that: (1) miR-494 is down-regulated in cancer; (2) miR-494 modulates multiple key players along the canonical G1 to S progression; (3) miR-494 induces a robust G1 arrest contributing, at least in part, to decreased cancer cell growth; and (4) miR-494 induces decreased tumor growth *in vivo*.

Many miR-based studies published to date focused on identifying pairs of interacting miR-mRNA. Although this approach brings invaluable information, it is of somewhat limited value in characterizing the global network regulatory effects of miRs. Furthermore, the effects of an miR species on the protein level of a target mRNA is usually modest, arguing that the interaction between one miR and one target is probably not sufficient to account for the effects of miRs on cell phenotype.[36] Therefore, miRs do not appear to function as on-off switches for any given target, but rather to function as rheostats to make fine-scale adjustments on multiple targets with a significant change in cell phenotype.[36]

The current data suggest that the manipulation of a sole miR species results in a significant phenotypic effect, such as decreased cancer cell growth. Except for CDK6, miR-494 exerted less than a 50% decrease in the level of the proteins tested (CDK4, CCND1, CCNE2, HDAC1, and phospho-RB). We believe that the effects of miR-494 are likely the end result of simultaneous action on multiple proteins along the same canonical pathway. Our findings further solidify the theory that miRs act as signaling pathway modulators, where relatively modest input may result in large responses.[37] Signaling pathways, which are highly dynamic, nonstoichiometric systems, with nonlinear dose-dependent responses, thus appear to be the ideal theater for miR function.[37]

The unbiased identification of regulatory molecules downstream of miR-494 by employing cDNA arrays, followed by western blot verification, unraveled a surprising coordination in the actions of miR-494. Our data suggest that miR-494 is a significant modulator of the G1-S transition canonical pathway by controlling expression of proteins involved at multiple steps. Previous data showed that exposure of murine bronchial cells to benzo(a)pyrene (a known carcinogen) up-regulates several miR species, including miR-494, and increases the percent of cells in the G1 phase of cell cycle.[38] In the current study we demonstrate that miR-494 up-regulation in human cancer cells has a direct effect on cell cycle regulation. We further demonstrate that miR-494 directly interacts with the 3′UTR of CDK6 and results in a decrease of CDK6 at the protein level.

In the current article we report that miR-494 acts on multiple targets involved in the regulation of the

G1-S transition checkpoint. CDK6 appears to be a direct target, whereas the rest of the targets appear to be indirect. We believe that uncovering the rheostat qualities of miR-494 onto the G1/S transition checkpoint and understanding its downstream effectors sheds new light onto miR-dependent cell growth regulation. Understanding of the global phenotypic effects of, as well as pathway modulation induced by, miR-494 is therefore crucial.

We found that, whereas the up-regulation of miR-494 in cancer cells induced G1 arrest, in normal cells it did not. Our findings advocate for a nonlinear relationship between the level of miR-494 and its effects, consistent with its involvement in cell signaling pathways. Also importantly, the lack of a cell-cycle impact of miR-494 in normal cells is reassuring from the perspective of developing miR-494-based therapeutics for *in vivo* delivery.

A previously published article reported that miR-494 is up-regulated in human retinoblastoma compared to normal retina.[39] We report a down-regulation of miR-494 in human cholangiocarcinoma as well as functional implications. The fact that miR-494 appears to be up-regulated in retinoblastoma and down-regulated in cholangiocarcinoma is not singular in the microRNA literature. Previously published studies reported contradictory expression levels, and sometimes roles, for several miRs in cancers arising in different organs. We suspect, as others, that the role of miRs is tissue and/or organ and/or context specific. Such examples include miR-31,[40,41] miR-126,[42,43] and others.

The data presented here suggest that miR-494 may represent a valuable therapeutic strategy for CCA treatment. In contrast to the more widely studied small interfering RNA (siRNA) species, miRs may offer the added benefit of being intrinsic molecular species. We speculate that because miRs are naturally occurring species, identifying miRs with impact on cellular functions may be extremely useful for utilizing their built-in, multi-pathway effects. Although their pervasive effects on multiple mRNA species may be construed as detrimental in terms of potential off-target effects, we hypothesize that it is precisely this quality that makes miRs potent agents. In addition, we theorize that a fully developed miR-based anticancer therapeutic agent will be difficult to evade by cancer cells, specifically because of the wide impact of miRs on multiple molecules within the same pathway.

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REVIEW

Long non-coding RNAs and cancer: a new frontier of translational research?

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Tiling array and novel sequencing technologies have made available the transcription profile of the entire human genome. However, the extent of transcription and the function of genetic elements that occur outside of protein-coding genes, particularly those involved in disease, are still a matter of debate. In this review, we focus on long non-coding RNAs (lncRNAs) that are involved in cancer. We define lncRNAs and present a cancer-oriented list of lncRNAs, list some tools (for example, public databases) that classify lncRNAs or that scan genome spans of interest to find whether known lncRNAs reside there, and describe some of the functions of lncRNAs and the possible genetic mechanisms that underlie lncRNA expression changes in cancer, as well as current and potential future applications of lncRNA research in the treatment of cancer.

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Keywords: long non-coding RNAs; cancer; online databases; function

INTRODUCTION

Non-protein-coding RNAs (ncRNAs) are gaining the attention of researchers in many fields, and the number of published articles is exponentially growing.[1] MicroRNAs (miRNAs) belong to a small ncRNA group and are the most studied among ncRNAs; however, many more types of ncRNAs exist. In fact, tiling array and novel sequencing technologies have made available the transcription profile of the entire human genome, which showed a widespread transcription activity.[2] However, the extent of transcription (that is, whether ncRNAs are mainly localized in close proximity to protein-coding genes (PCGs) or widespread throughout the genome) and the function of genetic elements that occur outside of PCGs are still a matter of debate.[3–5] Moreover, by more traditional means, several researchers have cloned RNA transcripts whose nature is probably not to code proteins and that have a longer sequence than miRNAs do. These can be grouped under the classification of long ncRNAs (lncRNAs).

The human genome census reveals a striking predominance of non-coding regions (http://www.ncrna.org/statgenome/index.html?view = class&gid = hg18). In fact, PCG exons represent about 1.6% of the 3×10^9 base pairs of the human genome. Moreover, the number of PCGs is quite steady during evolution in metazoa (G value paradox), whereas the size of genomes tends to increase.[6] Conservation among genomes also occurs within intergenic regions, suggesting that these regions are important in the fundamental processes involved in life. Finally, the largest part of the human genome, about 46%, is made up of repetitive elements (such as transposons) that probably have been one of the driving forces of evolution.[7] It is worth mentioning that in most cases transposons do not code for proteins, and recently they have been found to be related to cancer processes.[8,9]

In this review, we focus our attention on lncRNAs that are involved in cancer. First, we will define lncRNA and present a cancer-oriented list of lncRNAs. Second, we will list some tools (for example, public databases) that classify lncRNAs or that scan genome spans of interest to find whether known lncRNAs reside there. Some of the databases can also be used to search for lncRNAs that are involved in a process or disease of interest (for example, cancer). Finally, we will describe some of the functions of lncRNAs, possible genetic mechanisms that underlie lncRNA expression changes in cancer, and current and potential future applications of lncRNA research in the treatment of cancer.

DEFINITION OF lncRNA

The most commonly used definition of lncRNA is an RNA molecule that is longer than 200 nucleotides and that is not translated into a protein. However, this definition may be too simple and does not take into account certain issues. First, the cutoff of 200 nucleotides was arbitrarily chosen and it was set more on the basis of RNA binding to silica columns during RNA purification rather than for its functional meaning.[2] Second, a PCG is usually defined as a transcript that contains an open reading frame (ORF) longer than 100 amino acids.[10] However, lncRNAs can contain ORFs longer than 100 amino acids and not necessarily synthesize polypeptides; plus, polypeptides shorter than 100 amino acids can be functional in organisms and are not by-products of canonical proteins.[11] Finally, and even more confounding, the same RNA can contain both PCGs and non-coding functions.[12–14] These issues demonstrate how little we currently know about ncRNAs (particularly lncRNAs) and how difficult it is to form a definition.

One updated definition that we agree with takes into account some of the aforementioned issues[15] and defines lncRNAs as RNA molecules that may function as either primary or spliced transcripts and do not fit into known classes of small RNAs, such as miRNAs, piwi-interacting RNAs and small nucleolar RNAs, or into classes of structural RNAs (for example, transfer RNAs, small nuclear RNAs and spliceosomal RNAs). The strengths of this definition are the absence of ORF restriction, given the fact that a RNA molecule can possess both coding and non-coding activities, and the absence of length restriction that was arbitrarily set. Other investigators have proposed bioinformatic tools to clarify or adjust

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the 100-amino acid ORF length cutoff to determine whether an RNA molecule codes for a protein (reviewed in Dinger *et al.*).[10] The strengths of Mercer's definition are the absence of ORF restriction, as a matter of fact a RNA molecule can possess both coding and non-coding activities, and the absence of length restriction that was arbitrarily set.

Additionally, we must point out that in this review we use the abbreviation lncRNA, which should not be confused with long intergenic ncRNAs (lincRNAs)[16,17], which are a subtype of lncRNAs.

CLASSIFICATION OF LNCRNAS AND PUBLIC DATABASES

Generating comprehensive classifications of lncRNAs is not an easy task. In fact, many lncRNA classifications are annotations from larger databases or projects (for example, GeneBank, Fantom3), and information about the real nature (protein-coding, non-protein-coding or mixed) and function of lncRNAs cannot be gleaned from these sources. Similarly, some lncRNAs have been described in only one published study and no further reports exist.[18,19] Some lncRNAs have been grouped on the basis of their position relative to host PCG (for example, overlapping RNA, cis-antisense RNA, antisense RNA, bidirectional RNA, intronic RNA, promoter- or enhancer-correlated RNA).[15] As it usually happens for all classifications, the same lncRNA may be listed under different groups. For example, lncRNAs predicted by computational models (for example, RNAz or Evofold) are often listed under different names in databases obtained from sequencing projects.

To facilitate the difficult task of organizing lncRNAs, we have listed the current online databases that include ncRNAs (Table 1). These databases collect lncRNAs originated from GenBank annotations or from published articles. Some of these databases list both ncRNAs that have been experimentally proven and those that are purely computational predictions (based on RNA Z or Evofold) or have been annotated as ncRNAs on the basis of the predicted size of their ORFs.

We found the functional RNA project database (fRNA) worth visiting. It uses a University of California Santa Cruz genome browser interface that contains many ncRNA tracks that have already been set up in a Genome Browser graphic interface, which allows the user to search for specific ncRNAs along with other features in the genomic context of interest. Although both fRNA and the Noncode project allow the user to search for functional classes or processes (for example, find all known ncRNAs that are involved in the cell cycle or in DNA replication or transcription), fRNA allows the user to search by disease (for example, cancer) as well. The ncRNA expression database, on the other hand, contains a large data set of ncRNA expression profiles that were obtained from three different experiment sets: Allen Brain Atlas (mouse), GNF Atlas (mouse and human) and V1.0 Compugen array (mouse). Although these expression data sets are not cancer-oriented, we foresee that eventually the ncRNA expression database, as well as others that are listed in Table 1, will be matched with other data sets that are more cancer-oriented (for example, Oncomine; https://www.oncomine.org). For now, the genomic positions of several lncRNAs can be matched to databases that collect lists of single-nucleotide polymorphisms (SNPs) associated with cancer (http://cistrome.dfci.harvard.edu/CaSNP/; Hindorff *et al.*)[172] or cancer-associated genetic regions (for example, http://cancergenome.nih.gov, http://decipher.sanger.ac.uk, the Cancer Workbench at https://cgwb.nci.nih.gov/cgi-bin/heatmap and National Center for Biotechnology Information Gene Expression Omnibus at http://www.ncbi.nlm.nih.gov/geo/).[14,27]

CANCER-RELATED LNCRNAS

In this review, we focused our efforts on developing a list of lncRNAs that have been linked to cancer by various means. We mainly used three of the online databases to retrieve lncRNAs (that is, the LncRNA database, Noncode and the RNA Database), and we searched Pubmed for articles linking these lncRNAs to cancer. In Table 2, we report our findings.

In some cases the link between the lncRNA and cancer was obvious, and cancer was actually the model where these lncRNAs were first described for the first time (for example, MALAT-1,

Table 1. Public ncRNA databases

Website (reference)	*Name*	*Species*	*MicroRNA*	*Small nucleolar RNA*	*Infrastructural RNA (ribosomal RNA, transfer RNA, small nuclear RNA)*	*Notes*
http://biobases.ibch.poznan.pl/ncRNA/		Multiple kingdoms	Excluded	Excluded	Excluded	
http://www.noncode.org/ [20]	Noncode	Multiple kingdoms	Included	Included	Small nuclear RNA excluded	Experiment-based, ncRNAs divided on the basis of function (pf classes) and disease association
http://research.imb.uq.edu.au/rnadb/ [21]	Rnadb	Multiple kingdoms	Included	Included	Excluded	
http://www.ncrna.org/ [22]	fRNA	Multiple kingdoms	Included			Functional ncRNA catalog, microarray info about ncRNA, dedicated UCSC page
http://escience.invitrogen.com/ncRNA/		Human, mouse	Excluded	Excluded	Excluded	
http://rnaqueen.sysu.edu.cn/ncRNAimprint/ [23]	ncRNA imprint	Mammals	Included	Included	Excluded	Focused only on imprinting ncRNAs
http://jsm-research.imb.uq.edu.au/nred/cgi-bin/ncrnadb.pl [24]	NRED	Human, mouse	Excluded	Excluded		Expression data
http://www.lncrnadb.org/ [25]	lncRNAdb	Multiple kingdoms	Excluded	Excluded	Excluded	
http://rfam.sanger.ac.uk/ [26]	Rfam	Multiple kingdoms	Included	Included		

Abbreviations: fRNA, functional RNA; lncRNA, long non-coding RNA; ncRNA, non-protein-coding RNA; UCSC, University of California Santa Cruz. The website, reference and content are listed for each database, along with the most interesting feature of each website.

Table 2. lncRNAs that have been or might be (*) linked to cancer

lncRNA	*Molecular mechanism*	*Tumor*	*Reference*	*Genome position*	*Website*
aHIF	Messenger RNA decay	Multiple cancers	28–30	hg19 chr14:61,283,843–61,285,036	Noncode
Air	Epigenetic regulation	*	31	NA	lncRNAdb
ak023948	Unknown	Papillary thyroid carcinoma	32	hg18 chr8:134136386-134139194	lncRNAdb
alpha 250/ alpha 280	Antisense, transcription regulation	*	33,34	hg19 chr5:149,828,969-149,829,248	lncRNAdb
anril	Antisense, transcription regulation	Prostate cancer	35,36	hg18 chr9:21,984,790-22,111,091	lncRNAdb
anti-NOS2A	NA	Central nervous system tumors	37	hg18 chr17:57,692,139-57,696,081	lncRNAdb
antisense tgf beta 3	NA	*	38,39	NA	lncRNAdb
BA318C17.1	NA	Colon cancer	40	hg19 chr20:14,864,899-14,910,132	Rnadb
bc200	Protein binding	Multiple cancers	41,42	hg19 chr2:47,562,454-47,562,653	lncRNAdb
car intergenic 10	Regulation of gene expression	*	43	hg18 chr10:127,690,946-127,693,326	lncRNAdb
ccnd1-associated ncrnas	Regulation of gene expression	*	44,45	hg19 chr11:69,453,875-69,455,874	lncRNAdb
dhfr upstream transcripts	Regulation of gene expression	*	46	hg18 chr5:79,985,935-79,986,521	lncRNAdb
e2f4 antisense	NA	*	47	NA	lncRNAdb
emx2os	Antisense-sense pairing Dicer1	*	48–50	hg18 chr10:119,233,794-119,294,569	lncRNAdb
gas5	Decoy of glucorticoid receptor	Breast cancer	51	hg18 chr1:172,099,662-172,103,748	lncRNAdb
GNAS1-as RNA	NA	*	52–56	hg19 chr20:57,393,804-57,425,958	Noncode
h19	Transcription regulation (contains miR-675)	Multiple cancers	57–59	hg18 chr11:1,972,982-1,975,641	lncRNAdb
h19 antisense	Regulation of gene expression	*	60	NA	lncRNAdb
h19 upstream conserved 1 and 2	NA	*	61	NA	lncRNAdb
His-1 RNA	NA	*	62,63	hg19 chr2:145,456,944-145,465,439	Noncode
HOTAIR	Epigenetic regulation	Multiple cancers	64–66	hg18 chr12:52,642,363-52,648,782	lncRNAdb
hotairm1	NA	*	67–69	hg18 chr7:27,102,268-27,106,109	lncRNAdb
Hoxa11 antisense	NA	*	70–73	hg19 chr7:27,225,048-27,228,956	Noncode
hoxd3as	NA	*	74,75	NA	lncRNAdb
HULC	Micro RNA decoy	Multiple cancers	76–78	hg18 chr6:8,597,441-8,599,080	lncRNAdb
krasp1	Micro RNA decoy	Prostate cancer	14	hg18 chr6:54,743,128-54,743,996	lncRNAdb
KvlQT1-AS (Kcnq1ot1)	DNMT1 interaction and transcription gene silencing	Colon cancer	79	hg19 chr11:2,465,330-2,870,445	Noncode
LEU2	Pri-micro RNA, other	Chronic lymphocytic leukemia	80	hg19 chr13:50,556,688-50,699,677	lncRNAdb
LOC285194	NA	Osteosarcoma	81	hg18 chr3:117,911,325-117,918,575	lncRNAdb
LUST	RNA–RNA interaction, RNA splicing	*	19	hg18 chr3:50,112,040-50,113,425	lncRNAdb
MALAT-1 (NEAT2)	RNA splicing, small RNA production, protein interaction	Multiple cancers	82–84	hg18 chr11:65,021,809-65,030,513	lncRNAdb
MEG3	NA	Multiple cancers	85,86	hg18 chr14:100,362,198-100,397,121	lncRNAdb
MER11C	RNA–protein interaction, regulation of gene expression	Cell lines	83	hg18 chr11:50,410,308-50,411,367	lncRNAdb
Msx1 antisense	NA	*	87–90	NA	Noncode
ncR-uPAR	RNA–protein interaction	*	18	hg18 chr5:76,043,519-76,044,442	lncRNAdb
NCRMS	NA	*	91	hg19 chr12:97,886,239-97,954,478	Noncode
NDM29	NA	Neuroblastoma	92	hg18 chr11:8,917,158-8,917,288	lncRNAdb

Table 2. (Continued)

lncRNA	*Molecular mechanism*	*Tumor*	*Reference*	*Genome position*	*Website*
NEAT1/TncRNA	RNA nuclear export, paraspeckle organization	*	93–96	hg18 chr11:64,946,845-64,950,577	lncRNAdb
Nkx2.2AS	NA	*	97,98	NA	lncRNAdb
NRON	NFAT nuclear trafficking, RNA-protein binding	*	99,100	NA	lncRNAdb
NSCLC B2	NA	*	101,102	hg19 chr6:11,192,694-11,205,944	Rnadb
NTT sense/antisense	NA	*	103,104	hg19 chr6:136,265,389-136,282,959	Noncode
p53 mRNA	RNA protein binding	Multiple cancers	12	hg19 chr17:7571720-7590863	lncRNAdb
p53int1	NA	*	105	hg19 chr17:7,588,578-7,589,689	Rnadb
PCA3/DD3	NA	Prostate cancer	106	hg18 chr9:78,569,172-78,592,305	Noncode
PCGEM1	NA	Prostate cancer	107	hg18 chr2:193,322,816-193,349,870	Noncode
PCNA-AS	NA	*	108	hg19 chr20:5,100,232-5,100,615	Rnadb
PINC	NA	*	109	NA	lncRNAdb
PR Antisense	Regulation of gene expression	*	110	hg18 chr11:100,505,018-100,574,851	lncRNAdb
PRINS	NA	*	111,112	hg18 chr10:24,576,060-24,584,981	lncRNAdb
PTENP1	Micro RNA decoy	Prostate cancer	14	hg18 chr9:33,663,502-33,667,418	lncRNAdb
RMRP	Mitochondrial RNA processing endoribonuclease, hTERT-dependent small interfering RNA pathway	Leukemia and lymphoma	113,114	hg19 chr9:35,657,750-35,658,014	Rnadb
RPS6KA2 antisense transcript	NA	Cell lines	115	NA	lncRNAdb
saf	NA	Cell lines	116	hg19 chr10:90,751,179-90,752,732	Rnadb
SRA RNA	RNA-protein binding, transcription factor co-activator	Breast cancer	117,118	hg19 chr5:139,930,090-139,937,036	Noncode
TERC	Telomere template	Multiple cancers	119	hg19 chr3:169,481,881-169,483,646	Noncode
terra	Telomerase regulation	Multiple cancers	120,121	NA	lncRNAdb
tie-1AS	RNA–RNA interaction	*	122–124	NA	lncRNAdb
Tsix	Antisense of Xist	*	125,126	hg18 chrX:72,928,765-72,965,791	lncRNAdb
UBE3A antisense	NA	*	127,128	hg19 chr15:25,264,182-25,299,063	Noncode
uca1	NA	Bladder cancer	129,130	hg19 chr19:15,939,757-15,946,226	Rnadb
WT1-AS	NA	*	131	hg18 chr11:32,413,861-32,418,212	lncRNAdb
xist	X inactivation	Multiple cancers	125,132–134	hg19 chrX:73,043,280-73,072,588	Noncode
Zeb2NAT	NA	*	135,136	hg18 chr2:144,992,452-144,995,153	lncRNAdb
Zfas1	NA	Breast cancer	137	hg19 chr20:47,894,715-47,905,797	lncRNAdb

Abbreviations: lncRNA, long non-protein coding RNA; NA, not available. For each lncRNA, the name, function, tumor model in which it has been evaluated, and genomic position are listed, along with the public website where each lncRNA can be found.

PCA3/DDR3 and HOTAIR). However, we also found some lncRNAs for which a link to cancer has not yet been fully elucidated, but preliminary findings indicate that it could be worthwhile to investigate the possible connection (these lncRNAs are marked with an asterisk in Table 2). For example, the lncRNA Air (antisense to Igf2r RNA) is involved in the imprinting of the Igfr2 locus.[31] Despite the association of Igfr2 with cancer[138] and the association of Air with Igfr2,[139] we did not find any articles that directly examine the relationship between Air and cancer in humans. Moreover, although alpha 250/alpha 280 lncRNA regulates RPS14 transcription, which has been shown in short hairpin RNA screens to be a causal factor in 5q- syndrome,[33] no studies have yet examined the direct involvement of alpha 250/alpha 280 lncRNA in 5q-syndrome.[140]

Some lncRNAs that we included contain small ncRNA (for example, miRNA and small nucleolar RNA). Although these lncRNAs host small RNAs, this may not be their exclusive function. For example, the knockout model of LEU2, which includes miR-15/16 as well, showed a more aggressive phenotype than did the miR-15/16 knockout model, which may indicate

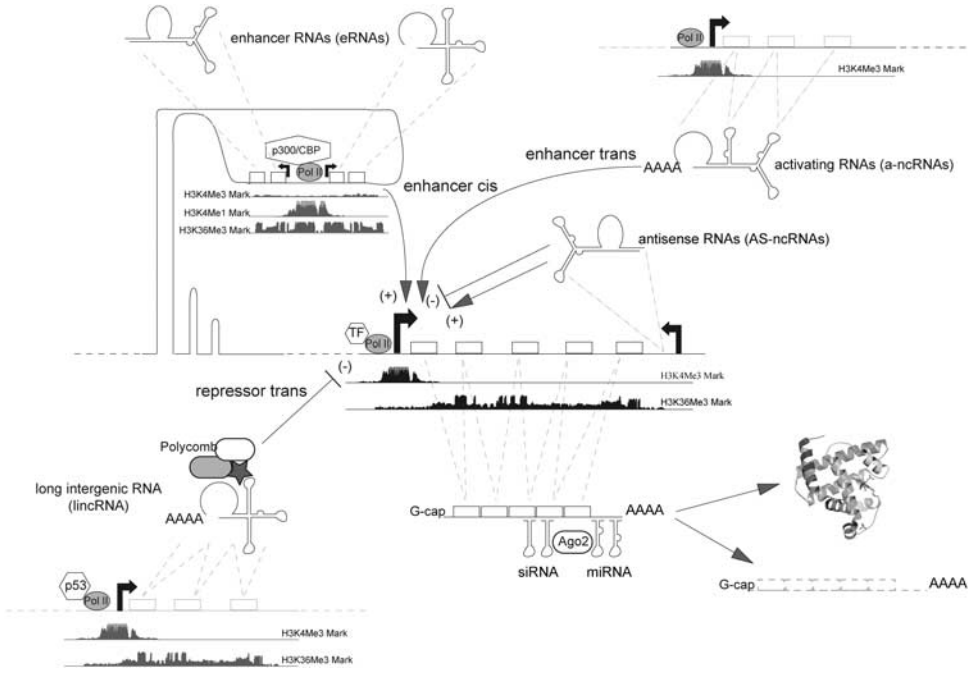

Figure 1. LncRNA categories and functions. Several classes and functions of lncRNAs are depicted. The main function of lncRNA seems so far to regulate PCG transcription; indeed, lncRNA can either enhance or repress PCG transcription by changes in the chromatin state of the PCGs (for example, by histone methylating or acetylating). Enhancer RNAs derive from transcription of enhancer elements that can be located several kilobases upstream of target genes. Enhancer DNA can both regulate gene expression by DNA looping and direct DNA–DNA interaction with the target promoter, and they also transcribe non-polyA RNAs (that is, eRNA). The function and the role of eRNAs is at this moment unknown. Overall, both long ncRNAs (lincRNA, a-ncRNAs and AS-ncRNAs) and small ncRNAs (for example, siRNA and miRNA) regulate transcription and post transcription steps of protein synthesis, respectively. At the bottom of coding and non-coding transcription units that are shown in picture, the reader can find the peak diagram for CHIP-seq experiments concerning histone modifications: H3K4Me1, mono methylation at lysine 4 of histone 3 (often found near regulatory elements); H3K4Me3, tri methylation at lysine 4 of histone 3 (often found near promoters); H3K36Me3, tri methylation at lysine 36 of histone 3 (often found near active transcripts).

that LEU2 can participate in chronic lymphocytic leukemia development.[80]

Some lncRNAs have been associated with cancer but are not listed in the public data sets that we used to prepare Table 2. For example, regions that are extremely conserved among human, mouse and rat genomes[141] are expressed in cancer tissue differently than in normal tissues and are regulated by methylation as well.[142–146] The extremely high level of conservation among these lncRNAs, which are referred to as ultraconserved genes or transcribed UCRs, is their most peculiar feature.

LNCRNA FUNCTION

The function of ncRNAs is the most difficult and least understood aspect of ncRNA research. Better understanding ncRNA function will help clarify the real impact of genomic pervasive transcription on cell biology and evolution.[147] As we gathered information about the lncRNAs involved in cancer, we also collected examples of lncRNA function (Figure 1).

The first example that we describe is for lincRNAs. LincRNAs were first described using histone mark signatures, specifically trimethylation in lysine 4 and lysine 36 of histone 3 (H3K4m3, H3K36m3 or simply K4K36). The K4K36 mark detects active transcription units of both PCGs and ncRNAs. After excluding known genes (PCGs and ncRNAs), researchers have been able to retrieve novel transcriptional units. The first reports analyzed mouse and human cell lines, uncovering about 3000 lincRNAs.[16,148] However, many more lincRNAs may remain to be discovered in other settings.[149] Certain, lincRNAs were discovered before the use of the K4K36 signature such as MALAT-1[82] and HOTAIR, which was the first lncRNA ever described to interact with polycomb proteins and suppress gene transcription.[64] Moreover, other histone signatures might reveal new lncRNAs.[150]

About 20% of lincRNAs bind to polycomb repressive complex 2, indicating that lincRNAs might regulate gene expression by directing the polycomb protein group to target DNA regions, inducing changes in histone marks and chromatin structure and ultimately suppressing transcription activity.[148,151] The current model proposes that lincRNAs directly bind to the polycomb proteins and direct them to specific DNA segments in the human genome. However, how the lincRNA-polycomb complex recognizes the target DNA is not currently known.[152] We do not currently know whether transcription factors bind lincRNAs as well, and whether RNA-binding proteins regulate lincRNAs as they do with miRNAs.[153]

Another class of lncRNAs that seems to regulate gene expression by changes in chromatin status includes antisense transcripts (reviewed in Morris and Vogt).[154] Antisense ncRNA transcripts overlap PCG but are transcribed in the opposite direction. Although one would expect small interfering RNA (siRNA) machinery to degrade messenger RNA after the sense–antisense pairing, the mechanism in act instead seems to be the modifications of histone marks at the promoter region of the sense transcript (that is, PCGs). Apparently, antisense lncRNAs drive (cytosine-5)-methyltransferase 3A (DNMT3A) to the DNA of the host PCG to methylate histones at lysine 9 and 27 or CpG islands and ultimately silence transcription.

Several oncogenes or tumor-suppressor genes exhibit antisense transcription and consequent transcription gene silencing (for example, *p21*, *c-Myc*, *p15*, *p53*, *TlE1* and *PU.1*).[35] Interestingly,

exogenous siRNAs that are in antisense orientation compared with PCG promoters are also effective at silencing transcription.[155] However, how the antisense lncRNAs are regulated has not yet been explored.

LincRNAs, antisense lncRNAs, and other lncRNAs[44,156] can be classified among the chromatin-associated RNAs (CARs) because their function apparently relies on the ability of the RNA to somehow bind to genomic DNA and consequently regulate chromatin states (euchromatin versus heterochromatin).[44,156] Mondal *et al.*[43] performed a thorough investigation of CARs throughout the genome of a human skin fibroblast cell line by deep sequencing of DNA-associated RNA after micrococcal nuclease treatment. They identified several CARs and reported that one CAR can activate transcription of neighboring genes.

Another class of lncRNAs that seems to regulate the transcription activity of host PCGs comprises the promoter upstream transcripts (PROMPTs). PROMPTs are localized upstream of promoters of some PCGs and they can be transcribed in both the sense and antisense orientations. PROMPTs seem to be a byproduct of RNA pol II activity; however, preliminary findings suggest that PROMPTs control promoter methylation.[45] When Preker *et al.* first described PROMPT existence, they used a peculiar approach: they inhibited exosome key proteins by using siRNA to prolong the half-life of short-lived RNA transcripts. In this way, they were able to identify a plethora of PROMPTs. However, the function and impact of PROMPTs in cell biology have not yet been explored.

It is possible that lincRNAs, PROMPTs, and antisense RNAs, or CARs in general, have interdependent functions. For example, antigene RNAs are synthetic RNA molecules that when designed to be complementary to PCG promoters can either repress or activate gene expression. Antigene RNAs rely on RNA–RNA interaction with antisense transcripts that are generated nearby targeted promoters and on Ago proteins binding.[157] It is possible that PROMPTs, lincRNAs and antisense lncRNAs interact and recapitulate antigene RNA mechanism; it is known that PROMPTs and antisense lncRNAs can interact with each other to trigger the antigene RNA pathway.[158] In another example of lncRNA interdependent function, lincRNAs can interact with PROMPTs or antisense lncRNAs to ultimately direct polycomb protein complexes to targeted promoters of PCGs.[36] Further examples of lncRNA function are discussed in other reviews.[159,160]

LNCRNA NETWORKS

Another interesting lncRNA function is target decoy or mimicry: lncRNAs can deceive another RNA or protein away from its natural target. For example, Poliseno *et al.*[14] described pseudogenes as decoys for miRNAs. They reported that the *PTEN* gene and the PTEN pseudogenes (PTENP1) share a high degree of sequence homology and are targeted by the same miRNAs (that is, miR-17, -21, -214, -9 and -26 families). Thus, changes in PTENP1 expression levels indirectly affect PTEN expression levels by sequestering *PTEN*-targeting miRNAs. For instance, if PTENP1 expression levels decrease, miRNAs will be able to target *PTEN* and ultimately downregulate PTEN expression levels. Poliseno *et al.*[14] also noted a similar mechanism for RAS pseudogenes. Another example of a lncRNA that acts as a miRNA decoy is the highly upregulated liver cancer transcript (HULC), which binds to and inhibits miR-372.[76]

Target decoys occur not only in cancer but also in infectious diseases: two studies reported that virus-encoded transcripts can act as miRNA decoys; in this case the net effect was to sequester and downregulate the miRNAs of the host organism.[161,162] A similar example exists in plants for endogenous pseudogene transcripts that share a high degree of sequence homology with PCG transcripts, although in this case the pseudogenes contain point mutations within the miRNA-binding sites. Apparently, these pseudogenes not only sequester miRNAs from their PCG target, but also reduce miRNA expression levels.[163]

One particular type of lncRNA decoy involves proteins. PROMPTs, such as GAS5, can bind to transcription-factor proteins that would otherwise bind to the DNA promoters; thus, the RNA transcript decoy sequesters the transcription factor, which is no longer able to affect downstream target genes.[164] GAS5 accomplishes this with a stem-loop structure in its sequence resembling the glucocorticoid receptor DNA-binding element. GAS5 seems to regulate other receptors (that is, androgen, mineralcorticoid and progesterone) by the same means. Interestingly, the interaction between GAS5 and the glucocorticoid receptor is modulated by dexamethasone, a glucocorticoid receptor agonist.[164] At the same time, GAS5 has been shown to be regulated by mammalian target of rapamycin pathway and to mediate rapamycin effect on cell cycle in T cells (reviewed in Williams *et al.*).[165]

NcRNA decoys can target not only ncRNA–mRNA or DNA–protein interactions, but also interactions between ncRNAs. For example, miRNAs can target other ncRNAs as they do with messenger RNA; Calin *et al.*[144] showed that miR-155 targets transcribed UCRs in both *in vitro* models and chronic lymphocytic leukemia patients. These findings support the existence of networks among ncRNAs and between ncRNAs and PCGs that are involved in cancer.

LNCRNA EXPRESSION IN CANCER

In cancer biology, one of the first evidences that researchers seek is gene expression differences between tumor and normal samples. The breadth of knowledge concerning lncRNA expression profiles in tumor and normal samples is quite modest at this time. It is likely that commercial gene expression arrays that have been used for PCGs contain probes that hybridize to lncRNAs, and it may be possible to retrieve cancer-related lncRNA expression profiles from public, tumor-specific gene-expression data sets (for example, Oncomine, Gene Expression Omnibus). However, to our knowledge this has not yet been done.

To identify novel transcripts, some investigators have used the Affymetrix tiling array, which can test for lncRNA gene expression.[166,167] Others have performed custom array profiling on large sample sets of a few lncRNAs.[144,168] Most articles concerning lncRNA expression in cancer have shown a selected number of lncRNAs probed in tumor samples (Table 2 lists tumor types that have been tested for lncRNA expression). We also found a few articles (not included in Table 2) reporting the existence of transcriptionally active regions that are located outside known PCGs and are differentially expressed between normal and tumor tissues or are expressed under stress conditions.[166,167] Gibb *et al.* used SAGE library generation to compare lncRNA expression in normal and dysplastic oral mucosa.[169]

Cancer biologists also seek to uncover genetic mutations (for example, amplifications, deletions and sequence mutations) in the lncRNA sequence. For example, sequence mutations in RNA component of mitochondrial RNA processing endoribonuclease (RMRP) lncRNA are responsible for cartilage-hair hypoplasia syndrome, which is also known to increase the risk of developing several types of tumors.[170,171] Some investigators have already sequenced select classes of lncRNAs to find mutations.[166,172]

In recent years, using SNP arrays to study very large populations (in the thousands), researchers have discovered several SNPs that are associated with certain traits or diseases, such as cancer (http://www.genome.gov/gwastudies contains a list of SNPs associated with several diseases).[173] In some cases, disease-associated SNPs are in genomic spans outside of PCG transcripts;[174,175] these genomic spans would be good candidates regions to search for novel transcripts. Some researchers have already found SNPs that are located within lncRNA transcripts and are associated with cancer. For example, Yang *et al.* showed that among six SNPs that are located within the boundaries of UCRs,

two of them (that is, rs2056116 and rs9572903) were significantly associated with familial breast cancer.[176] Cabili *et al*.,[27] while reporting on a census of 8195 lincRNAs in 24 different human tissues, noted that the genomic positions of 414 lncRNAs were related to SNPs that have been associated with several diseases.[173]

DIAGNOSTIC AND THERAPEUTIC APPLICATIONS OF LNCRNAS

The relatively new field of lncRNA research is expanding quickly, but many gaps still need to be filled. Only recently has the number of lncRNAs in the human genome become clear.[27] Moreover, researchers have not extensively investigated lncRNA expression in large and clinically controlled tumor data sets, nor is lncRNA function well understood.[149] Few examples of transgenic models of lncRNA have been published to date.[80,177]

We foresee potential uses of lncRNAs in the clinical setting for oncology or for other fields. LncRNAs may be useful as novel biomarkers for diagnosis, prognosis and prediction of response to therapy. The lncRNA PCA3/DD3, for example, has already been assayed in controlled clinical settings. PCA3/DD3 was originally discovered in a differential display analysis comparing normal and tumor prostate samples.[178] The features that make PCA3/DD3 a promising biomarker are its unique expression profile in prostate tumors compared with normal prostate and other tissues, its highly increased expression levels (that is, about 60 times) in prostate tumors compared with normal tissues, its expression in early-stage tumors and detectability in urine. PCA3/DD3 has been tested as a biomarker in clinical trials and compared with standard prostate markers (that is, prostate-specific antigen). However, the effectiveness of PC3/DD3 as a biomarker was about the same as that of prostate-specific antigen.[106,179]

The marked increase or decrease in lncRNA expression levels in tumors compared with normal tissues seems to be a feature shared among lncRNAs. Indeed, HOTAIR was found to be upregulated by hundreds or thousands of times in metastatic breast cancer tissue compared with normal breast tissue.[64] Such a large difference in lncRNA expression levels in tumors compared with normal tissues is a topic for future clinical research, although lncRNAs must be assayed in larger clinical data sets. Other lncRNAs might be promising biomarkers as well.[106,179]

Another potential avenue of lncRNA research relates to the discovery of circulating miRNAs in serum, plasma and other body fluids, demonstrating that miRNAs may act not only within cells, but also at other sites within the body.[180] It is highly probable that other types of ncRNAs, including lncRNAs, can be present in body fluids, as suggested by, for example, their presence in the secreted exosomes. LncRNAs found in numerous quantities in body fluids could be detected using simple quantitative reverse transcriptase polymerase chain reaction. This could represent an unexpected and yet unexplored gold mine of potential biomarkers predictive of survival or response to therapy.

LncRNAs might also be useful as therapeutic agents. The small size of miRNAs offers an intrinsic advantage in their use as therapeutic bullets by *in vivo* administration.[181] However, because lincRNAs are much longer than miRNAs, they could not be used directly as therapeutic bullets but would require gene therapy delivery systems (for example, viruses), which would carry potential risks. On the other hand, lncRNAs could be targeted with synthetic siRNAs or miRNAs. Another way to target lncRNAs would be with drugs designed specifically to interact with lncRNAs, as vault RNAs naturally do. Vault RNAs belong to the largest ribonucleoprotein complex in eukaryotic cells (that is, vault), and they are involved in multidrug resistance.[182] Gopinath *et al*. showed that vault RNAs directly bind to chemotherapeutic agents, indicating that it would also be possible to design small molecules that interact with lncRNAs. Of course, vault RNAs are technically short RNAs, ranging from 80 to 90 nucleotides; however, examples of longer RNAs involved with drug interaction exist, such as aptamers.[183–186]

Targeting transcripts the size of lncRNAs may seem like a daunting task, but there is a precedent for fragmenting large ribonucleoprotein complexes into more manageable sizes. This strategy has been applied in the design of ligands that can bind to expanded rCUG and rCAG repeat RNAs that are expressed in myotonic dystrophy type 1 and interact with the Muscleblind-like 1 protein.[187] Moreover, systematic evolution of ligands by exponential enrichment (SELEX) approach can be used to identify chemicals that interact with lncRNAs.[160]

As well as being potential markers or therapeutic targets, lncRNAs could be used as models to develop novel strategies to target tumor cells. For example, synthetic RNA molecules that form hairpin structures simulating DNA transcription factor-binding elements can be generated to target and regulate transcription factor activity as GAS5 does.[164] Synthetic lncRNAs that contain mutant miRNA-binding sites can sequester and reduce expression levels of miRNAs, as it happens in plants.[163]

Finally, small molecule compounds could be used to target lncRNAs. Indeed, small molecule compounds have already been tested for other uses in clinical trials to determine toxicity, body distribution and pharmacokinetics, and in some cases, their use in humans is already approved by the US Food and Drug Administration. Their use with lncRNAs requires only identifying, either by *in silico* predictions or by large library screens, the small molecules that target lncRNA or ribonucleoprotein complexes. If such compounds exist, the transition time from lab to clinic would be very short, which would be good news not only for scientists, but especially for patients with cancers and other diseases.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Decoy activity through microRNAs: the therapeutic implications

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Introduction: microRNAs (miRNAs), small noncoding RNAs, are deregulated in several diseases including cancer. miRNAs regulate gene expression at a post-transcriptional level by binding to 5′UTR, coding regions or 3′UTR of messenger RNAs (mRNA), inhibiting mRNA translation or causing mRNA degradation. The same miRNA can have multiple mRNA targets, and the same mRNA can be regulated by various miRNAs.
Areas covered: Recently, seminal contributions by several groups have implicated miRNAs as components of an RNA–RNA language that involves cross-talk between competing endogenous RNAs through a decoy mechanism. We review the studies that described miRNAs as players in a biological decoy activity. miRNAs can either be trapped by competing endogenous RNAs or interact with proteins that have binding sites for mRNAs.
Expert opinion: The miRNA decoy functions have implications for the design of therapeutic approaches in human diseases, including specific ways to overcome resistance to drug therapy and future miRNA-based clinical trials design.

Keywords: mimic, regulation, sponge, therapeutic potential

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1. Introduction

Contrary to the case with protein-coding genes, there is a proportional increase between the number of noncoding transcripts (ncRNA, RNAs that do not codify for proteins but influence by various mechanisms their expression and function) and the complexity among the species [1,2], with the ratio of noncoding to coding sequences being about 47:1 in humans [3]. The use of massively parallel sequencing platforms of "next-generation sequencing," with use of the RNA sequencing (RNA-seq) application, revealed thousands of transcripts and opened the door to a comprehensive study of the nonprotein-coding transcriptome [4,5]. Up to 97% of the human genome consists of nonprotein-coding DNA [6], and noncoding transcripts comprise a heterogeneous group that includes, among other, microRNAs (miRNAs), small nucleolar RNAs (snoRNAs), infrastructural RNAs (ribosomal RNA [rRNA], transfer RNAs [tRNAs], and small nuclear RNAs [snRNAs]), long intergenic noncoding RNAs (lincRNAs), antisense transcripts, noncoding transcribed ultraconserved regions, and promoter upstream transcripts (PROMPTs) [7]. The ncRNAs can be intergenic or can overlap with the coding regions loci, being antisense or intronic [2]. The paradigm of RNA as a mere intermediary between DNA and protein became obsolete when it became evident that the previously called "junk" DNA [8] could be of major importance for biologic diversity [5] and may have key biological functions [9]. Although much still needs to be studied about the function of ncRNAs, the top of the iceberg is being revealed as we increase our understanding of the importance of ncRNAs in the regulation of gene expression.

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Article highlights.

- RNA transcripts that have the same specific miRNA binding sites can compete for miRNAs binding.
- miRNAs can be trapped by ceRNAs that function as natural miRNA sponges.
- miRNAs function as RNA decoys upon interaction with heterogeneous ribonucleoproteins, as hnRNP (that have binding sites for mRNAs).
- Decoy mechanisms involving miRNAs and the balance between levels of specific ceRNAs should be considered when designing new therapeutic approaches aiming to correct gene expression.
- The hypothesis that miRNA-based decoy mechanisms play a role in the resistance to therapy is worthy of being explored.
- For therapeutic approaches using precursor microRNAs, when two mature forms are processed, their expression, function and mRNA targets should be considered, as some of these could be ceRNAs for both 3p and 5p of that specific miRNA.

This box summarizes key points contained in the article.

In this review, we intend to focus on the studies that identified the miRNA decoy activity, and we propose to emphasize the potential significance of these studies for therapy.

2. Current evidence

miRNAs are small ncRNAs, approximately 20 nucleotides long, that control gene expression at a posttranscriptional level by mRNA degradation or translation inhibition, through the binding to 5′UTR, coding sequences, or 3′UTR of target mRNAs [10]. miRNAs can regulate multiple target genes, and simultaneously, target genes can be regulated by multiple miRNAs. Victor Ambros' and Gary Ruvkun´s groups first discovered miRNAs in 1993 as a new mechanism of gene regulation in *Caenorhabditis elegans* [11,12]. In 2000, Pasquinelli *et al.* proved that let-7 was conserved among species, opening the door for the study of miRNAs in humans [13]. Later, in 2002, it was reported that miRNAs were deregulated in cancer [14]. To date, thousands of studies have analyzed the expression of miRNAs and its role in cancer and in cardiovascular, autoimmune, and neurodegenerative diseases [10].

Recently, another function for miRNAs was found: the decoy activity. In early 2010, Perrotti´s group reported the paradigm shift in miRNA function [15]. hnRNP E2 is a member of the family of RNA-binding proteins whose members are involved in mRNA processing, nucleocytoplasmic export, and translation of mRNAs [16]. In cells in which the BCR/ABL oncogene is expressed, causing an arrest of differentiation in myeloid blast crisis chronic myelogenous leukemia, there is an increase of hnRNP E2 protein [17]. hnRNP E2 interacts with the transcription factor *CEBPA*, inhibiting the translation of mRNA. Using RNA electrophoretic mobility shift assays, UV crosslinking, and RNA immunoprecipitation assays, the authors found that miR-328 (that is downregulated in a BCR/ABL-dependent manner) competes with *CEBPA* mRNA for the hnRNP E2 binding site [15]. They also proved that restoration of miR-328 expression interferes with hnRNP E2 function of translation inhibition by preventing *CEBPA*:: hnRNPE2 binding and consequently restores, *in vivo* and *in vitro*, *CEBPA* mRNA translation [15] (**Figure 1A**). Besides the decoy activity, miR-328 also functions in the canonical way by suppressing translation of mRNA encoding the PIM1 protein kinase through base pairing interaction [15].

Later the same year, Poliseno *et al.* reported in *Nature* the intriguing discovery that pseudogenes could function as a decoy for miRNAs' effects on corresponding protein-coding genes [9]. The authors used as a model the well-known tumor suppressor *PTEN* and its pseudogene *PTENP1*, which has a high sequence homology with part of the *PTEN* 3′UTR [9]. The authors proved that *PTENP1* is targeted by some of the miRNAs that target also *PTEN*, including miR-19b and miR-20a. Through a miRNA-dependent mechanism, overexpression of *PTENP1* 3′UTR resulted in the derepression of *PTEN* (and consequently proved that *PTENP1* has a role as a tumor suppressor), and expression of *PTEN* 3′UTR resulted in the derepression of *PTENP1* [9]. In addition, the authors showed that the same decoy mechanism is present when analyzing other genes and their related pseudogenes, such as the *KRAS* gene and its pseudogene *KRAS1P* [9]. This new concept was further developed 1 year later, when the same group showed that not only noncoding genes can compete for miRNAs binding sites, but also protein-coding transcripts can compete with one another [18]. Transcripts that have the same miRNA binding sites (or miRNA response elements [MREs]) are called "competing endogenous RNAs" (ceRNAs) [19] and may act as natural miRNA sponges. The authors used bioinformatics (MRE enrichment—MuTaME—analysis) and biological approaches to validate ceRNA for *PTEN* [18]. Some of these mRNAs are *SERINC1*, *VAPA*, and *CNOT6L*, whose expression in human prostate cancer and glioblastoma samples was significantly different between PTEN-high and PTEN-low groups [18]. In addition, silencing of these ceRNAs resulted in a decrease in luciferase activity when cells were co-transfected with a luciferase vector containing the *PTEN* 3′ UTR. The authors further proved that this correlation was dependent on the miRNAs, since regulation of *PTEN* expression by *SERINC1*, *VAPA*, and *CNOT6L* ceRNAs vanished in the cells with a defect in the miRNA processing machinery [18] (**Figure 1B**).

In the same issue of *Cell*, three articles reported "out-of-the-box" discoveries about coding transcripts and competing endogenous RNAs. Sumazin *et al.* used a multivariate analysis method, named Hermes, to combine gene expression data with miRNA profiles in glioblastoma and found 7,000 genes whose transcripts were involved in sponge regulatory interactions (modulator and sponges share miRNAs binding sites) and 148 genes that were involved in nonsponge regulatory interactions (modulator and sponges that do not necessarily share miRNA binding sites) [20]. Moreover, the authors focused on the *PTEN* transcript to perform validation

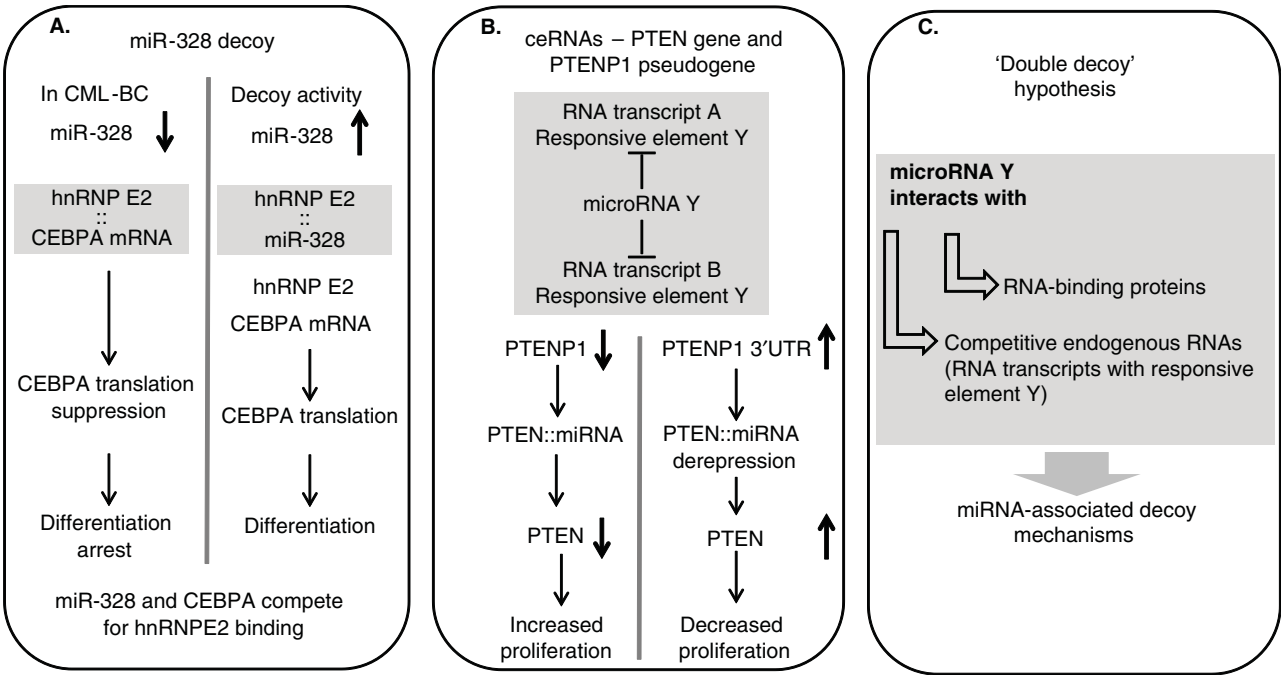

Figure 1. The decoy by microRNAs. A. miR-328 decoy. In blast crisis chronic myelogenous leukemia (CML-BC), miR-328 is downregulated. The RNA binding protein hnRNP E2 interacts with *CEBPA* mRNA, suppressing it translation and causing a differentiation arrest. When miR-328 restoration is induced, miR-328 interacts with hnRNP E2, releasing *CEBPA* from the translation inhibitory effects of hnRNAP E2 and leading to *CEBPA* mRNA translation. **B**. Complementary endogenous RNAs (ceRNAs) – *PTEN* gene and pseudogene. The basic principle of ceRNA is that different RNAs (e.g., RNA transcript A and RNA transcript B) that contain the same microRNA binding sites (e.g., responsive element Y) can compete with each other for those microRNAs (e.g., microRNA Y). The first example of ceRNAs was the *PTEN* gene and it pseudogene *PTENP1*, that share same microRNA responsive elements. When *PTENP1* is silenced, the tumor suppressor PTEN is decreased leading to an increase in cell proliferation. Accordingly, when *PTENP1* 3ÇUTR is overexpressed, PTEN levels increased due to a decoy for the microRNAs, causing a decrease in cell proliferation. **C**. The hypothesis states that the same miRNA can be trapped between binding to proteins and to ceRNAs. This represents a combination of the two experimentally identified instances presented in (**A**) and (**B**). When miRNA Y interacts with an RNA binding protein, the effect of this interaction on the competing mRNA species could be variable. In the case of miR-328 and hnRNP E2, the interaction of miRNA Y with the protein induces mRNA translation. However, one could hypothesize that interaction of miRNA Y with a protein that stabilizes mRNAs and induces translation (e.g. hnRNP A1) could result in reduced expression of mRNA containing the same miRNA binding sites.

studies for *PTEN* miRNA-mediated decoy in glioblastoma cell lines [20].

In another study, Karreth *et al.* identified *PTEN* ceRNAs in a mouse model of melanoma with use of the sleeping beauty transposon system [21]. The authors further validated *ZEB2* as a ceRNA decoy for *PTEN* by demonstrating that ZEB2 depletion downregulates PTEN and that this reduction was dependent on the 3′UTR (ZEB depletion suppressed luciferase activity of *PTEN* 3′UTR reporter) and on miRNAs (ZEB2 depletion does not reduce PTEN expression in cells with a defect in the miRNA processing machinery) [21]. Finally, Cesana *et al.* reported that a long noncoding RNA, *linc-MD1*, acts as a ceRNA for *MAML1* and *MEF2C* mRNAs, two transcription factors that regulate muscle-specific genes, by interacting with miR-133 and miR-135, thereby regulating muscle differentiation [22].

All of the above-mentioned studies were crucial to a new understanding of the importance of miRNAs in mediating mRNA decoys, and their significance has been highlighted in several articles. For example, McCarthy [23] stated that a subtle reduction in few mRNA could cause widespread effects. Rigoutsos and Furnari [24] indicated that the relative amount of mRNAs and corresponding ceRNAs should be considered and they introduced the intriguing hypothesis that the decoy mechanism may also occur in cases in which PTEN levels are reduced without mutation, such as in Cowden disease and Bannayan-Zonana syndrome. In addition, Swami [25] questioned how this decoy applied in noncancer-related genes. In the "Expert Opinion" section, we give our opinion from a therapeutic point of view.

3. Expert opinion: potential therapeutic consequences

The capacity of multiple mRNAs to compete for available miRNAs introduces a new challenge for miRNA-based

cancer therapies because the expression levels of the several transcripts regulated by the miRNAs need to be specifically taken into account. miRNAs have been suggested not only as therapeutic targets in several diseases but also as therapeutic tools because miRNAs can regulate the protein expression of major genes involved in disease and therefore influence patient outcome. There are two major strategies to modulate miRNA expression and consequently influence gene expression: miRNA antagonists (e.g., to knock down miRNA oncogenic activity) and miRNA mimics (e.g., to restore miRNA tumor suppressor activity). The decrease in miRNA levels may be achieved by using chemically modified antisense oligonucleotides such as 2′-O-methyl, 2′-O-methoxyethyl, morpholino oligomers and locked nucleic acid (LNA) [26]. All of these molecules function as miRNA sponges, since they are designed to be complementary to miRNAs and to sequester miRNAs and derepress the mRNA targets. Studies have indicated that research using LNAs (first described in the late 1990s) is quickly progressing. miR-122 reduction using LNA (complementary with miR-122 sequence) was tested in primates [27]. Lanford *et al.* successfully suppressed chronic hepatitis C virus infection in chimpanzees by using LNA against miR-122 and obtained long-lasting but no secondary effects [27]. In contrast to the chemically engineered antagomirs, ceRNAs function as natural biological sponges. In the case of *PTEN* and its pseudogene *PTENP1*, if we could therapeutically overexpress the pseudogene, *PTEN* would be automatically derepressed since miRNAs would bind mainly to the pseudogene. If we could modulate the levels of ceRNAs, we could restore the expression of a full network of mRNA through miRNAs interaction. It is therefore crucial, when deciding on a therapeutic approach involving miRNAs, to consider not only the miRNA levels in that specific cancer, but also the expression balance between the levels of the ceRNAs. In addition, not only the amount of transcripts but also the stability of the miRNA::mRNA binding should be analyzed. Furthermore, in light of the Perrotti group's publication, the same miRNA could bind both ceRNAs and also proteins with binding sites for specific mRNAs (maybe themselves ceRNAs). This introduces another level of complexity since, for example, hnRNPs are members of a large family of proteins that are usually highly overexpressed in human cancers [15] and therefore could easily trap the interactor miRNAs (**Figure 1C**).

It is well known that miRNAs are involved in drug resistance [28]. An unexpected consequence of the ceRNA concept is the fact that resistance to therapy can be caused by an abnormal balance between the ceRNA network components. For example, the cancer-specific overexpression of pseudogene(s) of a gene involved in blocking the activity of a drug can "trap" miRNA molecules that otherwise in normal cells would bind to and downregulate the specific drug-resistance gene. In this case, the gene involved in blocking the activity of a drug would be translated causing drug resistance. Indeed, if a miRNA that targets genes inducing drug resistance is trapped by ceRNAs, it is expected that the mRNAs involved in drug resistance are derepressed, which would contribute to increased resistance to specific drugs. Also, the opposite mechanism can be postulated – the release of miRNAs that target genes involved in the induction of cell death by the drug, due to downregulation of ceRNAs. This will lower the malignant cell sensitivity to drug with consequent increased cell survival and resistance to particular chemotherapeutic regimens. Therefore, in cancer cells, the interactor miRNAs are "used" by the interaction with the multiple ceRNA messengers instead of directly targeting and consequently downregulating the coding genes (**Figure 2**). The mechanisms of ceRNA expression variation are the well-known ones for any coding or noncoding gene: genomic deletions or amplifications, chromosome translocations, loss-of-function or gain-of-function mutations or promoter methylation. Of course, in the absence of experimental proof on the therapeutic consequences of ceRNAs, this is a hypothesis, but one well worth exploring!

Finally, another intriguing possibility is the fact that the two distinct miRNAs generated from the same hairpin precursor can function as a decoy if they share also common targets. This could profoundly influence the design of clinical studies involving miRNA-based therapy. Although studies involving miRNA inhibitors are already being developed in primates, efforts to apply miRNA mimics to therapy are progressing more slowly. One important aspect to consider when designing therapeutic approaches for miRNA mimics is the fact that precursor miRNAs can generate two different mature miRNAs, -5p and -3p, depending on the hairpin stem-loop strand of the precursor miRNA, which may have cooperative or different functions. This is the case of miR-28, which we recently reported to be downregulated in colon cancer [29]. We reported that miR-28-5p, but not miR-28-3p, affects colon cancer cell proliferation by increasing apoptosis and causing G1 arrest. On the other hand, miR-28-5p decreases while miR-28-3p increases colon cancer cells migration and invasion. These differential effects are partially explained by the different mRNA targets. *In vivo*, we observed that miR-28 expression disrupts tumor growth but increases metastasis [29]. Furthermore, we identified several common mRNA targets that are predicted to interact in independent sites for 5p and 3p miR-28, sometimes separated only by few nucleotides. Similarly, Jiang *et al.* reported that miR-125a-5p and miR-125a-3p, which are downregulated in non-small cell lung cancer, have opposite effects *in vitro* on invasion and migration in cells of this tumor type [30]. Consequently, when designing vectors using precursor miRNA, it is essential to consider the function of both -5p and -3p mature forms because one of the strands may cause adverse effects. Furthermore, the main challenge for miRNA mimics will be the *in vivo* delivery strategies. Since the ceRNA mechanism suggests the existence of multiple miRNAs that target simultaneously the same spectrum of coding genes and pseudogenes/other ncRNAs, the 5p/3p pairs of miRNAs could represent examples of such decoy miRNAs.

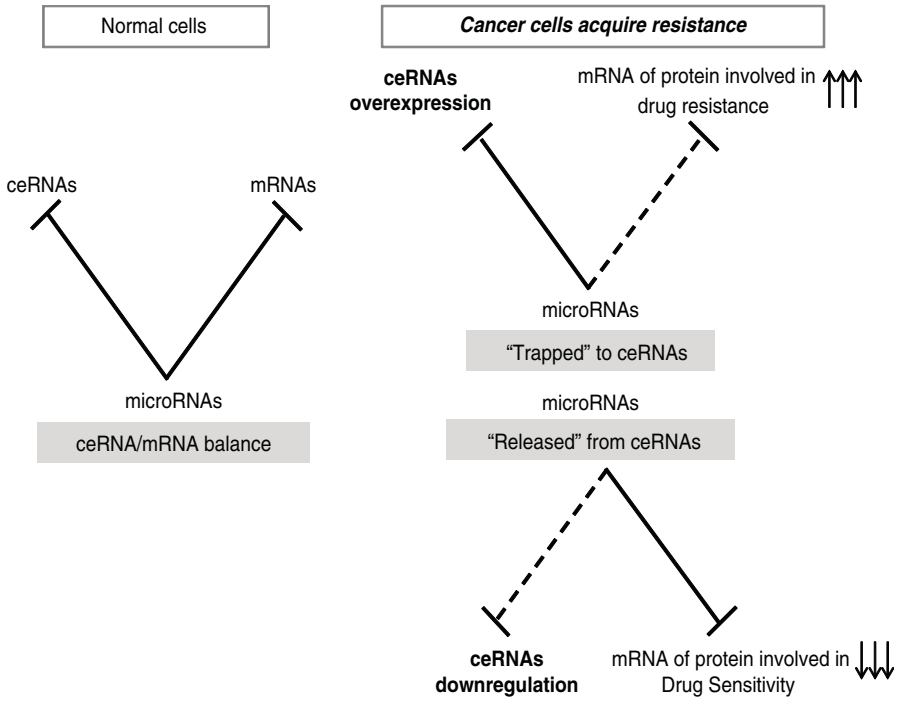

Figure 2. Schematic hypothesis of the involvement of ceRNA/miRNA/mRNA in resistance to therapy. Under normal conditions, there is a balance between the "RNA language" components: ceRNAs, miRNAs, and mRNAs. In cancer, cells may overexpress ceRNAs (with the same miRNA-binding sites as mRNAs involved in drug resistance), which will trap the available miRNAs, causing a derepression of mRNAs involved in drug resistance. This will consequently cause overexpression of drug-resistant mRNA levels and therefore contribute to increased resistance to drug treatment. Other possibility is that in cancer cells specific ceRNAs are downregulated with consequent "release" of one or more miRNAs that could target messengers of genes involved in proper activity of a specific drug. The consequence is that sensitivity to the drug decrease. Note that in this model, the actual levels of the specific miRNA(s) did not change; what change is the ratio between the various targets and consequently the availability of "free" miRNA molecules ready to interact. Of course, the outcome could be further influenced by intrinsic variations in the expression of the specific miRNA (overexpression or downregulation).

4. Conclusion

One of the main challenges for application of miRNA-based therapies is the *in vivo* delivery method. In most cases, the delivery should be tissue-specific (and in some cases, even cell type-specific); in addition, the secondary undesirable effects should be controlled and the effect produced should be stable and long-lasting. Furthermore, the ceRNAs network and the expression levels of ceRNAs should be considered when designing the therapeutic approach for a particular miRNA-target gene.

In conclusion, it seems to be a question of time until miRNA-based therapies can be used to treat cancer as well as cardiovascular, inflammatory, and neurodegenerative diseases. Until then, efforts should be made to solve all of the above-mentioned issues. It is expected that in the next few years, similar to what happened in the miRNAs field, a second boon in the long noncoding RNAs field will emerge and additional functions for these genes will be revealed. This is the case, for example, of regions in the genome that have a high level of homology and conservation between species and whose ultraconserved transcripts have been shown to be deregulated in cancer [31] but whose function has not yet been described. Certainly, the efficiency of ceRNA effects *in vivo* and the simplicity of this powerful concept remind us that the ncRNA El Dorado is a land where both scientists and clinicians should step without dogmas set in stone, but instead should be ready to accommodate new ideas that could benefit the people who need them at most —the patients!

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Declaration of interest

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Modulation of MicroRNA-194 and Cell Migration by HER2-Targeting Trastuzumab in Breast Cancer

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Abstract

Trastuzumab, a humanized monoclonal antibody directed against the extracellular domain of the HER2 oncoprotein, can effectively target HER2-positive breast cancer through several mechanisms. Although the effects of trastuzumab on cancer cell proliferation, angiogenesis and apoptosis have been investigated in depth, the effect of trastuzumab on microRNA (miRNA) has not been extensively studied. We have performed miRNA microarray profiling before and after trastuzumab treatment in SKBr3 and BT474 human breast cancer cells that overexpress HER2. We found that trastuzumab treatment of SKBr3 cells significantly decreased five miRNAs and increased three others, whereas treatment of BT474 cells significantly decreased two miRNAs and increased nine. The only change in miRNA expression observed in both cell lines following trastuzumab treatment was upregulation of miRNA-194 (miR-194) that was further validated *in vitro* and *in vivo*. Forced expression of miR-194 in breast cancer cells that overexpress HER2 produced no effect on apoptosis, modest inhibition of proliferation, significant inhibition of cell migration/invasion *in vitro* and significant inhibition of xenograft growth *in vivo*. Conversely, knockdown of miR-194 promoted cell migration. Increased miR-194 expression markedly reduced levels of the cytoskeletal protein talin2 and specifically inhibited luciferase reporter activity of a talin2 wild-type 3′-untranslated region, but not that of a mutant reporter, indicating that talin2 is a direct downstream target of miR-194. Trastuzumab treatment inhibited breast cancer cell migration and reduced talin2 expression *in vitro* and *in vivo*. Knockdown of talin2 inhibited cell migration/invasion. Knockdown of trastuzumab-induced miR-194 expression with a miR-194 inhibitor compromised trastuzumab-inhibited cell migration in HER2-overexpressing breast cancer cells. Consequently, trastuzumab treatment upregulates miR-194 expression and may exert its cell migration-inhibitory effect through miR-194-mediated downregulation of cytoskeleton protein talin2 in HER2-overexpressing human breast cancer cells.

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Introduction

HER2 is amplified and overexpressed in 25–30% of human breast cancers. Treatment with trastuzumab (Herceptin®), a humanized murine anti-HER2 monoclonal antibody [1], produced an objective response rate of 15% in extensively pretreated patients [2] and in 26% of previously untreated women with HER2-overexpressing metastatic breast cancer [3]. Trastuzumab in combination with chemotherapy was significantly more effective than chemotherapy alone in prolonging the time to disease progression, duration of response and overall survival [4,5,6,7], resulting in its approval by the FDA in 1998. Trastuzumab provided one of the first deliberately targeted therapies for a human cancer and has now become the first-line treatment of choice for patients with primary or recurrent HER2-overexpressing breast cancer [8].

Trastuzumab-based combination treatment with chemotherapy are, however, effective in 60–70% of the HER2-overexpressing breast cancers [7,9] and trastuzumab resistance often develops during the course of treatment [9,10]. Improving response to trastuzumab and reducing trastuzumab resistance is a critical and urgent priority for improving the clinical management of a substantial subset of patients with breast cancer. While significant progress has been achieved, molecular mechanisms underlying the action of trastuzumab are still not fully elucidated. Treatment with trastuzumab inhibits proliferation of breast cancer cells by blocking the phosphatidylinositol-3-kinase (PI3K) signaling pathway, upregulating the cyclin dependent kinase inhibitor p27Kip1 and inducing G1 arrest of the cell cycle [11,12,13,14,15,16,17,18,19,20]. We and others have also illustrated the ability of trastuzumab to interfere with DNA repair [14,21] and tumor angiogenesis [22,23,24,25,26,27]. Others have provided evidence that trastuzumab can cleave the extracellular

domains of HER2 receptors [28,29], mediate antibody-dependent cellular cytotoxicity [30,31,32], and can induce apoptosis [33,34,35].

Despite these many observations, the effects of trastuzumab on regulation of cellular processes by microRNA (miRNA) are still not investigated in depth. miRNAs are small non-coding RNAs of 19–25 nucleotides that can negatively regulate gene expression at post-transcriptional and transcriptional levels [36,37]. The binding of miRNAs to complementary sites in the 3′- untranslated regions (UTRs) and other regions of protein-coding mRNA sequences cause either degradation of the mRNA or inhibition of translation [36,37]. miRNAs have been demonstrated to regulate many normal physiological processes [36,37], to participate in malignant transformation at many sites including breast cancer [38,39,40], and to mediate sensitivity to chemotherapy and radiotherapy [41,42,43]. In this report, we have measured the effect of trastuzumab on miRNAs and the role of miRNAs in trastuzumab-mediated regulation of human breast cancer cells that overexpress HER2. We have found that treatment with trastuzumab affected expression of several miRNAs. miR-194 was upregulated and downregulated talin2, inhibited cell migration and invasion, which may contribute to the anti-tumor activity of trastuzumab on HER2-overexpressing breast cancer cells.

Materials and Methods

Cell lines and cell culture

Human breast cancer cell lines BT474, SKBr3, MDA-MB-361 and MDA-MB-231 were originally purchased from the American Type Culture Collection (Manassas, VA) and stored, recovered and used at early passage from cryopreservation in liquid nitrogen. SKBr3, MDA-MB-361 and MDA-MB-231 cells were maintained in RPMI 1640 medium containing 10% fetal bovine serum (FBS), 2 mM L-glutamine, penicillin (100 units/mL), and streptomycin (100 μg/mL). BT474 cells were grown in complete medium containing DMEM supplemented with 10% FBS, 2 mM L-glutamine, 1 mM sodium pyruvate (Sigma, St. Louis, MO), 100 units/ml of penicillin, and 100 μg/ml streptomycin. All cell culture media were obtained from the Media Preparation Core Facility at the University of Texas MD Anderson Cancer Center. All cell lines were cultured in a humidified air supplemented with 5% CO_2 at 37°C and tested monthly for mycoplasma with a MycoSensor PCR Assay Kit from Stratagene (Cat# 302109, La Jolla, CA). All cell lines were authenticated by the Cell Line Core Facility at the University of Texas MD Anderson Cancer Center with short tandem repeat DNA fingerprinting in April, 2011.

Reagents

Trastuzumab (Herceptin®) manufactured by Genentech Inc (South San Francisco, CA) was purchased from Division of Pharmacy at the University of Texas MD Anderson Cancer Center. Human immunoglobin G (hIgG) served as control for trastuzumab and was purchased from Calbiochem (La Jolla, CA). Antibodies reactive with vinculin and total Rb were purchased from Cell Signaling Technology, Inc. (Beverly, MA). Antibody against talin2 was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). miR-194 precursor and its miRNA negative controls, miR-194 inhibitor and its negative inhibitor, and TaqMan assay for miR-194 were purchased from Applied Biosystems Incorporated (ABI, Foster City, CA). Small interfering RNAs (siRNAs) targeted to talin2 and talin1 were from Dharmacon (Lafayette, CO) or Ambion (Austin, TX). Epidermal growth factor (EGF) used in cell culture was ordered from Sigma (St. Louis, MO). Transfection reagents used were Lipofectamine 2000 from Invitrogen (Grand Island, NY) and DharmaFECT #4 from Dharmacon.

Preparation of Total RNA

Total RNA was extracted with the TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. RNA purity was assessed by measuring absorption at 260 nm (A260) and at 280 nm (A280) (samples that had A260/A280 ratio of 1.9–2.1 were considered acceptable) and by ethidium bromide staining of 18S and 28S RNA on gels after electrophoresis. RNA concentrations were determined from the A260. Before performing the microRNA assay, each RNA sample was treated by DNase with a Turbo DNase kit (Ambion, Austin, TX).

miRNA Microarray Profiling

miRNA profiling was performed in the non-coding RNA core facility at M.D, Anderson Cancer Center. Briefly, 5 μg of RNA from each tissue sample was labeled with biotin by reverse transcription using random octomers in duplicate. Hybridization was carried out on the second version of a miRNA-chip A-MEXP-1246 version (http://www.ebi.ac.uk/arrayexpress/arrays/A-MEXP-1246), which contained 559 human and 393 mouse miRNA probes, 962 human ultraconserved sequences in quadruplicate. Each oligo was printed in duplicate in two different slide locations. Hybridization signals were detected by biotin binding of a Streptavidin-Alexa647 conjugate (one-color signal) using a GenePix 4000B scanner (Axon Instruments). We quantified images using the GenePix Pro 6.0 (Axon Instruments). Raw data were analyzed in BRB-ArrayTools developed by R. Simon and A.P. Lam (version: 4.2.1, National Cancer Institute). Expression data were normalized by quantiles method of the Bioconductor package. Statistical comparisons were done using BRB class comparison *t*-test. A total of 16 samples from BT474 cells (8 from wild-type or trastuzumab-sensitive cells and another 8 from trastuzumab-resistant cells) and 8 samples from SKBr3 cells (4 from wild-type or trastuzumab-sensitive cells and another 4 from trastuzumab-resistant cells) were analyzed. Samples were discarded if more than 87.5% and 75% for BT474 and SKBr3 cell line, respectively, of missed values occurred (the miRNAs are retained when present in at least the smallest group in the dataset), missed values were replaced with 4.500001 (basic expression value, log2) after normalization. miRNAs significant at 0.05 level of the univariate test were reported. This microRNA microarray data regarding breast cancer cell lines treated with or without trastuzumab have been deposited into the ArrayExpress (http://www.ebi.ac.uk/miamexpress/).

Quantitative Reverse Transcription–Polymerase Chain Reaction (QRT-PCR) Analysis

QRT-PCR was performed to assess mir-194 levels with the use of a Prism 7900HT Sequence Detection System (ABI, Foster City, CA) and TaqMan Real-Time PCR Assay (ABI). Total RNA was reversely transcribed into complementary DNA (cDNA) with specific stem-loop RT primer and a cDNA kit from ABI. Hsa-miR-194 was purchased from ABI (Assay ID 000493). Amplifications were carried out in triplicate on MicroAmp optical 96-well microliter plates (ABI). Thermal cycling conditions were as follows: 95°C for 5 minutes, followed by 40 cycles of 95°C for 10 seconds and 59°C for 40 seconds. U6 RNA was used as an internal control in all QRT-PCR assays for miRNA. The $\Delta\Delta C_T$ method was used to compare the relative expression levels between treatments. The

final PCR results were expressed as the relative expression compared to individual control sample in each assay.

RNA Blotting

miR-194 Northern blotting was performed as described previously [44]. Briefly, total RNA isolation was performed using the Tri-Reagent (Invitrogen). RNA samples of 25 μg each were run on 15% acrylamide denaturing (urea) criterion precast gels (Bio-Rad), and then transferred onto Hybond-*N*_membrane (Amersham Pharmacia Biotech). The hybridization with a DNA oligonucleotide probe complementary to the mature miR-194 sequence end-labeled with [γ-^{32}P] ATP was performed at 42°C in 7% SDS/0.2 M Na_2PO_4 (pH 7.0) overnight. The membrane was washed twice at 42°C with 2× standard saline phosphate EDTA (SSPE, 0.18 M NaCl/10 mM phosphate, pH 7.4/1 mM EDTA)/ 0.1% SD S and twice with 0.5× SSPE/0.1% SDS. The blot was stripped by boiling in 0.1% SDS/0.1× SSC for 10 min and was reprobed U6 snRNA as a loading control.

miRNA Transfection

BT474 or SKBr3 cells were seeded on 6-well culture plates and transfected with a negative control sequence or miR-194 using the DF4 reagent (Dharmacon). The mixture of miRNA (15 nM final concentration) and DF4 reagent (12.5 nM final concentration) were incubated for 20 min before being added to cells. Transfection was carried out for 72 hrs and cells were harvested for extraction of RNA or protein.

Cell Proliferation Assays

A crystal violet cell proliferation assay was used to assess anchorage-dependent cell proliferation as described previously [15]. Briefly, BT474 stable cells that either express empty vector or miR-194 were seeded and cultured in triplicate in 96-well cell culture plates for different intervals. The cells were washed with phosphate-buffered saline, fixed in 1% glutaraldehyde, and stained with 0.5% crystal violet (Sigma Chemical Co.) dissolved in methanol. The dye that stained the cells on the plates was then eluted with Sorenson's buffer (0.9% sodium citrate, 0.02 N HCl, and 45% ethanol), and directly measured with the use of a Vmax microplater reader (Molecular Devices, Sunnyvale, CA) at a wavelength of 560 nm. Cell number was expressed as the optical density (OD) at 560 nm.

BT474 Human Breast Cancer Xenografts in Nude Mice

The BT474 xenograft model was established in female nu/nu Balb/c mice as described previously [22] . Briefly, BT474 wild-type cells or stable transfectants expressing miR-194 were injected subcutaneously into two mammary fat pads of 4-week-old BALB/ c athymic Nu/Nu mice (obtained from the in-house animal facility at the Department of Experimental Radiation Oncology, the University of Texas MD Anderson Cancer Center). Experiments with nu/nu mice were reviewed and approved by the Institutional Animal Care and Use Committee at the University of Texas MD Anderson Cancer Center. All mice were maintained under specific pathogen-free conditions and given sterile food and water. Once the tumors became palpable (at day 14 after injection), the mice were randomly divided into two treatment groups (6 mice per group): 1) intraperitoneal injection with control hIgG (1 mg/kg) twice a week for 3 weeks.; or 2) intraperitoneal injection with trastuzumab (1 mg/kg) twice a week for 3 weeks. At the end of treatment, all tumors were collected and weighed. A portion of the xenografts was used for RNA and protein isolation; another portion was fixed in formalin and embedded in paraffin; and third portion was frozen in liquid nitrogen. Experiments with nude mice were repeated twice with similar results.

miR-194 Expression Construct

A miR-194 core fragment was amplified from BT474 genomic DNA by using the following primer set: CTAAGCTTAGTGGG-CATGGGACACTCT (miR-194-2F) & CTGAATT-CACCTGCCTCTCCTTCTTCGT (miR-194-2R) and sub-cloned into pEGPF-C1 vector digested with HindIII and EcoRI. The sequence of this miR-194 construct was verified by direct sequencing and QRT-PCR after transient expression.

Generation of Trastuzumab-resistant Cells and Stable Clones that Express miR-194 in BT474 and SKBr3 cells

Trastuzumab-resistant SKBr3 and BT474 cells were generated as reported previously [45]. Two stable clones that express miR-194 and two control clones that express the backbone vector (pEGPF-C1) were established in BT474 cells using previously reported methods [46] .

Generation of 3′ UTR reporter constructs of talin2

Prediction of miR-194 binding sites was performed using TargetScan software (http://www.targetscan.org/). A fragment of 3′-′UTR region of the talin2 contains the predicted binding site for miR-194 and was amplified by PCR using the primers: TCTAGAGGCTGCATGATCGTGATGT (forward) and TCTAGATCATAAAGAGGTCCAGGAGCA (reverse), which contained Xba I restriction sites (underlined nucleotides). The PCR product was purified, digested and cloned into pGL3 vector (Promega, Madison, WI) via the Xba I site, which is located downstream of the firefly luciferase reporter gene. QuikChange® II XL Site-Directed Mutagenesis Kit (Agilent Technologies, Santa Clara, CA) was used to generate a deletion mutation in the miR-194 seed region according to the manufacturer's instructions. Following mutagenic primers: 5′-GGGTGCACGTTTCATG-GACAACAAAGAAAAGTCAGT-3′ (deletion sense) and 5′-ACTGACTTTTCTTTGTTGTCCATGAAACGTGCACCC-3′ (deletion antisense) were utilized. Generated constructs were confirmed by direct sequencing using an ABI 3730xl DNA sequencer at the DNA Analysis Core Facility at the University of Texas MD Anderson Cancer Center.

Dual Luciferase Reporter Assay

Luciferase activity assays were performed as previously reported [46] . Briefly, cells were seeded in 6-well plates, cotransfected with miR-194 precursor or its negative control and a wild-type or mutated talin2 3′-UTR reporter construct as described above. A *Renilla* luciferase vector (pRL-TK) served as an internal control and was included in all samples. After transfection for 16 hrs, cells were split into 12-well plates, harvested after 24 hrs and Firefly and Renilla luciferase activities were measured sequentially using the dual luciferase assay kit (Promega) and a luminometer. Results were expressed as relative luciferase activity after normalization with *Renilla* luciferase activity. Results represent three independent experiments and each performed in triplicate.

Immunoblot Analysis

Total cell lysates were prepared and Western blotting was performed as described previously [15]. Briefly, cells were transfected with miR-194 precursors for 3 days, and then harvested for total lysate preparation. Total lysates were separated on 6% SDS-polyacrylamide gel and blotted onto nitrocellulose membrane. The membrane was incubated with horseradish

peroxidase–conjugated secondary antibody (1:2000; GE healthcare) and bound antibody was visualized with the use of a SuperSignal West Dura chemiluminescent kit (Thermo Fisher, Rockford, IL).

Flow Cytometry

The percentage of the sub-G1 cell population (apoptotic cells) and the cell cycle distribution were determined based on relative DNA content with the use of flow cytometry as described previously [15].

Cell Migration Assay

2×10^5 of BT474 cells or 5×10^4 SKBr3 cells in 0.5 ml of serum-free medium were introduced into the upper compartment of the BD BioCoat control inserts (Cat. # 354578, BD Discovery Labware, Bedford, MA) fitted with membranes of 8 micron porosity separating the upper and lower compartments. The lower compartment was filled with normal culture medium, medium supplemented with 10% FBS. After 16 hrs of incubation, cells were wiped off from the upper surface of each insert. The cells on the lower surface, which represented the cells that migrated through control insert membrance, were fixed and stained with Diff-Quick (Siemens, Deerfield, IL) and counted by microscopic examination in 10 representative fields. Cell migration was expressed as relative migration relative to the migration of each control group. Cell migration at each control group was arbitrarily set as 1. Each condition was assayed in triplicate and each experiment was repeated at least three times.

Cell Invasion Assay

Invasion assays were performed using BD Biocoat Matrigel Invasion Chambers (Cat. # 354480, BD Discovery Labware, Bedford, MA) that contain an 8 micron pore size PET membrane with a thin layer of MATRIGEL Basement Membrane Matrix by following the manufacturer instructions. Briefly, control-treated or miR-194 (or trastuzumab)-treated BT474 or SKBr3 cells were introduced into the upper compartment, incubated for 24 hrs, fixed and stained after removing non-invading cells as described above for the Cell Migration Assay. Cell invasion is then calculated as the percent invasion through the matrigel matrix and membrane relative the migration through the control membrane. Cell invasion at each control group was arbitrarily set as 1. Cell invasion data was expressed as relative invasion relative to the invasion of control group.

In Vitro Scratch Assay

BT474 (5×10^6 cells/well) or SKBr3 (2×10^6 cells/well) cells were seeded to 90% confluence in a 6-well plate for overnight culture. The following day a scratch was made through the center of each well using a 200-µL pipette tip, creating an open "scratch" or "wound" that was clear of cells. The dislodged cells were removed by three washes with complete culture media, and cells were incubated under standard conditions. Migration into the open area was documented at 72 hrs post-scratching.

Vinculin Immunofluorescence (IF) Staining

SKBr3 cells were seeded at 1.5×10^5 cells onto collagen (10 µg/ml) and poly-L-lysine (1 µg/ml) coated glass coverslips in 6-well dishes. Cells were grown for 16 hrs and then either cultured untreated or treated with control hIgG (10 µg/ml) or trastuzumab (10 µg/ml) for 16 hrs. Cells were fixed in ice-cold MeOH at -20°C for 5 min. Fixed cells were permeabilized and immunostained with rabbit anti-vinculin and followed by incubation with secondary antibody secondary antibody conjugated with Alexa Fluor 594 (Invitrogen). Coverslips were mounted on glass slides and examined using an Olympus FluoView FV1000 confocal microscope (Center Valley, PA).

Ethics Statement

All animal experiments with nu/nu mice were reviewed and approved by the Institutional Animal Care and Use Committee at the University of Texas MD Anderson Cancer Center. Animals were euthanized when the mice became morbid or the tumor size is 1.5 cm in the largest diameter.

Statistical Analysis

All experiments were repeated at least three times on different occasions. The results are presented as the mean ± SD for all values. A paired Student's t test was used to evaluate statistically significant differences in miR-194 levels between the treatment groups and the vehicle control group. $P<0.05$ was considered statistically significant. All statistical tests and corresponding P values were two-sided.

Results

Trastuzumab treatment upregulates miR-194 in HER2 overexpressing breast cancer cells

To determine the effects of trastuzumab treatment on miRNAs in HER2-overexpressing breast cancer cells, SKBr3 and BT474 cells were treated with trastuzumab or control hIgG. RNA was extracted and profiled on a miRNA chip (ArrayExpress) that contained 559 human miRNA probes and 962 human ultra-conserved sequences. Raw data were analyzed using a GeneSpring GX 7.3. The filter on fold change was set at 1.2 since this threshold had been demonstrated to reflect a significant biological difference [47]. As shown in Table 1, trastuzumab treatment in SKBr3 cells increased 23 human miRNAs including miR-194 and miR-629, but decreased 33 human miRNAs in SKBr3 cells. In BT474 cells, trastuzumab treatment upregulated more miRNAs than it downregulated. As shown in Table 2, trastuzumab increased 40 human miRNAs including miR-194, decreased 18 human miRNAs in BT474 cells. To visualize the clusters of the differentially expressed miRNAs in response to trastuzumab treatment in the two cell lines, hierarchical clustering was performed using one minus correlation as a distance measure and average linkage method for defining the distance between the clusters (Fig. S1). When miRNA changes were compared in SKBr3 and BT474 breast cancer cells, miR-194 upregulation was the only change shared by both cell lines. Consequently, miR-194 was selected for further study.

Trastuzumab upregulates miR-194 in cell culture and in xenografts

To confirm that miR-194 was upregulated by trastuzumab (Tables 1 & 2), total RNA was extracted from trastuzumab-treated SKBr3 and BT474 cells in culture and subjected to quantitative reverse-transcription PCR (QRT-PCR) and Northern blot analyses. QRT-PCR analysis confirmed that trastuzumab treatment increased miR-194 by 1.64 fold in SKBr3 cells (Fig. 1A) and by 1.95 fold in BT474 cells (Fig. 1A). Northern blotting further confirmed that trastuzumab treatment increased miR-194 level by 2.1 fold in BT474 cells (Fig. 1C). An assay with a miR-194-specific sensor reporter also indicated that trastuzumab increased miR-194 expression (data not shown). Consistent with above results in cell culture, QRT-PCR analysis of BT474 xenograft tumor samples

Table 1. miRNAs affected by trastuzumab in SKBr3 cells.

Unique ID	Mean ratio of trastuzumab vs control hIgG	p-value (t-test)	Geom mean of intensities in SKBr3 cells treated with control hIgG for 20 hrs (BRC20)	Geom mean of intensities in SKBr3 cells treated with trastuzumab for 20 hrs (BRHCT20)
hsa-mir-548b-A	0.065146674	0.0002274	347.37	22.63
hsa-mir-551a-P	0.071772555	0.0185408	452.68	32.49
hsa-mir-624-A	0.072392834	0.0169737	312.6	22.63
hsa-mir-648-A	0.075340413	0.0148338	300.37	22.63
hsa-mir-544-A	0.076597617	1.10E-05	295.44	22.63
hsa-mir-26a-2-P	0.083364032	0.0067642	271.46	22.63
hsa-mir-508-A	0.086219377	0.0030508	262.47	22.63
hsa-mir-608-P	0.099895462	0.028476	325.24	32.49
hsa-mir-33-A	0.100908274	0.0283768	300.57	30.33
hsa-mir-519a1-5p/526c-A	0.103456158	2.57E-05	218.74	22.63
hsa-mir-433-P	0.103754986	0.0014047	218.11	22.63
hsa-mir-548a3-P	0.110535828	0.033025	204.73	22.63
hsa-mir-516-4-5p-A	0.116595394	0.0157548	194.09	22.63
hsa-mir-520g-P	0.132984662	0.01343	170.17	22.63
hsa-mir-526a-2-P	0.135202744	0.0372519	247.85	33.51
hsa-mir-192-P	0.174889247	0.0003376	397.28	69.48
hsa-mir-519b-3p/526c-A	0.180074799	0.0152054	125.67	22.63
hsa-mir-141-P	0.202469357	0.0036423	111.77	22.63
hsa-mir-153-2-A	0.215605945	0.0208085	104.96	22.63
hsa-mir-381-A	0.240514401	0.0338008	94.09	22.63
hsa-mir-613-P	0.264091493	0.0242617	85.69	22.63
hsa-mir-135b-A	0.277634646	0.0053856	81.51	22.63
hsa-mir-506-P	0.314426262	0.0432334	199.22	62.64
hsa-mir-616-P	0.362195903	0.0017904	62.48	22.63
hsa-mir-651-A	0.381449112	0.0349512	212.82	81.18
hsa-mir-496-P	0.410558781	0.0179086	55.12	22.63
hsa-mir-652-A	0.45070703	0.045327	50.21	22.63
hsa-mir-624-P	0.477062244	0.0097793	139.29	66.45
hsa-mir-200a*-5p-A	0.509914376	0.0301038	44.38	22.63
hsa-mir-135a-1-A	0.558314088	0.0331035	103.92	58.02
hsa-mir-196b-P	0.56347169	0.0475296	2699.55	1521.12
hsa-mir-214-A	0.697566648	0.0485026	1745.74	1217.77
hsa-mir-29a-A	0.713695323	0.0199925	1379.23	984.35
hsa-mir-629-A	1.215502868	0.0165149	1799.28	2187.03
hsa-mir-194-2-A	1.222424312	0.0168992	3056.95	3736.89
hsa-mir-27b-A	1.466522781	0.0174398	838.63	1229.87
hsa-mir-572-P	1.710230649	0.0482883	706.7	1208.62
hsa-mir-518a2-5p/mir527-A	2.068051259	0.0064793	22.63	46.8
hsa-mir-569-P	2.840919134	0.0007198	22.63	64.29
hsa-mir-17-3p-A	2.933274414	0.0304101	22.63	66.38
hsa-mir-431-A	3.683163942	0.0495408	22.63	83.35
hsa-mir-30d-P	3.899322034	0.0487109	29.5	115.03
hsa-mir-96-P	4.094521778	0.022245	44.54	182.37
hsa-mir-557-P	4.207247017	0.0134777	22.63	95.21
hsa-mir-105-2-A	4.314184711	<1e-07	22.63	97.63
hsa-mir-489-P	4.814453843	0.0087891	54.38	261.81
hsa-mir-505-P	5.205695142	0.0310113	29.85	155.39

Table 1. Cont.

Unique ID	Mean ratio of trastuzumab vs control hIgG	p-value (t-test)	Geom mean of intensities in SKBr3 cells treated with control hIgG for 20 hrs (BRC20)	Geom mean of intensities in SKBr3 cells treated with trastuzumab for 20 hrs (BRHCT20)
hsa-mir-583-A	5.604502889	0.0038414	50.19	281.29
hsa-mir-640-P	6.07512152	6.07E-05	22.63	137.48
hsa-mir-548d1-A	8.575879397	0.0167225	29.85	255.99
hsa-mir-519a1-3p-A	9.625276182	4.25E-05	22.63	217.82
hsa-mir-605-A	11.04639859	0.0011691	22.63	249.98
hsa-mir-340-P	11.06934673	0.0302754	29.85	330.42
hsa-mir-198-A	11.44410075	0.0041912	22.63	258.98
hsa-mir-19b2-A	12.41228458	0.0020803	22.63	280.89
hsa-mir-518e-3p-A	13.12019443	0.002743	22.63	296.91

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also showed that treatment with trastuzumab induced miR-194 expression by 3.1 fold compared to control hIgG (Fig. 1D). Collectively, above results confirm that trastuzumab induces miR-194 in cell culture and in vivo.

Trastuzumab specifically induces miR-194 expression in trastuzumab-sensitive breast cancer cells

As shown in Figure 1E, miR-194 expression increased only in trastuzumab-sensitive SKBr3 and BT474 parental cells but not in trastuzumab-resistant cells. Additionally, miR-194 was not induced by trastuzumab treatment in either the MDA-MB-231 breast cancer cell line that expresses low levels of HER2 and is insensitive to trastuzumab or the KPL4 breast cancer cell line that expresses high levels of HER2 and is insensitive to trastuzumab (Fig. S2). Thus, miR-194 is specifically induced by trastuzumab treatment and may associate with trastuzumab response.

Increased expression of miR-194 significantly inhibits migration and invasion of breast cancer cells that overexpress HER2

We next explored the biological function of miR-194 in breast cancer. Two approaches were used to study the function of miR-194 in breast cancer cells: transient and stable expression of miR-194 in BT474 or SKBr3 breast cancer cells. The effects of transient and stable expression of miR-194 on cell viability, cell cycle, apoptosis and cell migration/invasion were evaluated. Stable expression of miR-194 in subclones of BT474 cells was confirmed with QRT-PCR and shown in Figure 2A. As shown in Figure 2B, stable overexpression of miR-194 in two BT474 stable clones (#22 and #23) produced moderate inhibition of cell growth as measured with a crystal violet viability assay. No significant change in cell cycle distribution or apoptosis was observed in subclones of BT474 with stable overexpression of miR194 (data not shown). Transient overexpression of miR-194 significantly decreased the ability of SKBr3 cells to migrate (Fig. 2C). Compared with the negative control miRNA, miR-194 expression reduced SKBr3 cell migration by 56% (Fig. 2D). Transient overexpression of miR-194 also decreased the ability of SKBr3 cells to detach from and invade through the matrigel matrix as illustrated in the cell invasion assay (Fig. 2E). Validation of miR-194 transient expression was performed with QRT-PCR and shown in Figure 2F. Additionally, stable overexpression of miR-194 inhibited the ability of BT474 cells to migrate by 40% and to invade by 55% (Fig. 2G & 2H). The effect of miR-194 on HER2-overexpressing breast cancer cells was further evaluated in a BT474 xenograft model. As shown in Figure 2I, miR-194 expressing BT474 tumors grew significantly less rapidly than did tumors containing breast cancer cells with an empty vector. Taken together, these data indicate that miR-194 can significantly inhibit cell migration/invasion in vitro, and tumor growth in vivo in breast cancer cells that overexpress HER2.

miR-194 directly targets the talin2 gene and downregulates talin2 protein

We next asked whether miR-194 targeted the expression of proteins which regulate migration and invasion of breast cancer cells (Fig. 2). Based on the UCSC genome database at http://genome.ucsc.edu/, miR-194-2 maps on human chromosome 11 within an intron of an unknown human gene (AB429224, also called Homo sapiens cDNA FLJ35483). Using the TargetScan and miRanda programs, we identified talin2 that encodes a cytoskeletal protein as one of many genes targeted by miR-194. The putative miR-194 target site in the 3′-UTR of talin2 gene shows a close match to nucleotides 2 to 18 of miR-194 (Fig. 3A). The miR-194 target site is also highly conserved among multiple species including humans, chimpanzees, mice, rats, rabbits, hedgehogs, dogs, cats, horses and elephants (Fig. 3A). To determine whether miR-194 acts directly on talin2 3′-UTR, we conducted luciferase reporter assays, cotransfecting miR-194 and luciferase reporter constructs containing wild type (underlined letter in Figure 3A) or mutant (deletion of underlined letter in Figure 3A) talin2 3′-UTR. Luciferase activity was dramatically decreased by approximately 60% in the presence of miR-194 when compared with its negative miRNA control (Fig. 3B). In contrast, miR-194 did not alter activity of the mutant talin2 luciferase reporter that contained the deletion of miR-194 binding site (Fig. 3B), indicating miR-194 specifically act on wild-type talin2 3′-UTR. In agreement with the luciferase reporter results, transient overexpression of miR-194 significantly decreased talin2 protein expression (Fig. 3C). These data indicate that talin2 is a direct target of miR-194 in breast cancer cells.

Depletion of talin2 inhibits cell migration and invasion in breast cancer cells that overexpress HER2

We next asked whether talin2 regulates cell migration and invasion. Both talin1 and talin2 encode high-molecular-weight

Table 2. miRNAs affected by trastuzumab in BT474 cells.

Unique ID	Mean ratio of trastuzumab vs control hIgG	p-value (t-test)	Geom mean of intensities in BT474 cells treated with control hIgG for 48 hrs (BTC48)	Geom mean of intensities in BT474 cells treated with trastuzumab for 48 hrs (BTHCT48)
hsa-mir-211-P	0.092615377	0.034884	382.01	35.38
hsa-mir-514-2&3-A	0.10147527	0.000624	223.01	22.63
hsa-mir-518e-5p/526c-A	0.116523351	0.019672	194.21	22.63
hsa-mir-452*-3p-A	0.116631449	0.034182	194.03	22.63
hsa-mir-518a2-5p/mir527-A	0.144231995	0.035946	156.9	22.63
hsa-mir-524*-5p-A	0.162257116	0.00944	139.47	22.63
hsa-mir-518b-A	0.173503028	0.012805	130.43	22.63
hsa-mir-105-2-P	0.209385572	0.012188	458.15	95.93
hsa-mir-520h-P	0.227391479	0.002751	99.52	22.63
hsa-mir-519e*-5p-A	0.242584266	0.002037	331.39	80.39
hsa-mir-646-A	0.242915414	0.023069	93.16	22.63
hsa-let-7g-P	0.257715522	5.90E-06	87.81	22.63
hsa-mir-323-A	0.291360886	0.002132	77.67	22.63
hsa-mir-517b-3p-A	0.291698891	0.009441	77.58	22.63
hsa-mir-432-5p-A	0.292150788	0.010767	77.46	22.63
hsa-mir-515-1-5p-A	0.305356902	7.16E-05	74.11	22.63
hsa-mir-548a1-A	0.500150716	0.017816	663.5	331.85
hsa-mir-671-A	0.780467508	0.030883	1862.64	1453.73
hsa-mir-758-P	1.203505577	0.010282	790.74	951.66
hsa-mir-485-3p-A	1.226628339	0.007659	1019.29	1250.29
hsa-mir-102-A	1.238857151	0.007481	1353.11	1676.31
hsa-mir-202-3p-A	1.29220961	0.03779	964.65	1246.53
hsa-mir-629-A	1.363440315	0.017991	2721.96	3711.23
hsa-mir-663-P	1.371448683	0.003362	1647.98	2260.12
hsa-mir-560-P	1.446595051	0.041021	831.29	1202.54
hsa-mir-296-A	1.45630605	0.010238	545.27	794.08
hsa-mir-324-5p-A	1.462267846	0.032113	1455.39	2128.17
hsa-mir-219-1-P	1.517478105	0.000529	17165.19	26047.8
hsa-mir-206-P	1.594267196	0.000415	577.03	919.94
hsa-mir-498-A	1.630858004	0.019047	783.33	1277.5
hsa-mir-19b2-A	1.699504866	0.038757	351.42	597.24
hsa-mir-194-2-A	1.828481053	0.00689	3749.44	6855.78
hsa-mir-219-2-P	1.868439905	0.008312	1298.19	2425.59
hsa-mir-429-P	1.895429772	0.031979	1099.07	2083.21
hsa-mir-564-P	2.086289374	0.018767	875.89	1827.36
hsa-mir-521-2-P	2.461776403	0.010133	22.63	55.71
hsa-mir-126-5p-A	2.653743792	0.002951	1067.1	2831.81
hsa-mir-199a*-3p-A	2.856385329	0.048539	22.63	64.64
hsa-mir-101-1/2-P	3.303137428	0.000142	22.63	74.75
hsa-mir-638-P	3.462660186	0.04302	22.63	78.36
hsa-mir-548a3-P	3.467962881	0.000185	22.63	78.48
hsa-mir-571-A	3.785682722	0.000292	22.63	85.67
hsa-mir-643-P	3.855501547	1.14E-05	22.63	87.25
hsa-mir-662-P	4.589482987	0.009066	22.63	103.86
hsa-mir-559-P	4.655766681	7.55E-05	22.63	105.36
hsa-mir-584-A	5.155103844	0.030886	22.63	116.66
hsa-mir-585-P	6.66195316	0.018554	22.63	150.76

Table 2. Cont.

Unique ID	Mean ratio of trastuzumab vs control hIgG	p-value (t-test)	Geom mean of intensities in BT474 cells treated with control hIgG for 48 hrs (BTC48)	Geom mean of intensities in BT474 cells treated with trastuzumab for 48 hrs (BTHCT48)
hsa-mir-659-A	6.761655012	0.015715	85.8	580.15
hsa-mir-587-A	7.121078215	0.019825	22.63	161.15
hsa-mir-591-A	8.88054883	0.048778	37.17	330.09
hsa-mir-18b-P	8.956212523	0.023338	71.71	642.25
hsa-mir-152-A	13.43314501	0.036953	37.17	499.31
hsa-mir-642-P	17.71718957	0.006654	22.63	400.94
hsa-mir-21-P	17.86831639	0.004662	22.63	404.36
hsa-mir-567-P	22.46221829	0.007096	22.63	508.32
hsa-mir-337-A	24.40212108	0.008015	22.63	552.22
hsa-mir-652-A	27.14891737	2.90E-05	22.63	614.38
hsa-mir-136-A	32.47459125	0.005502	22.63	734.9

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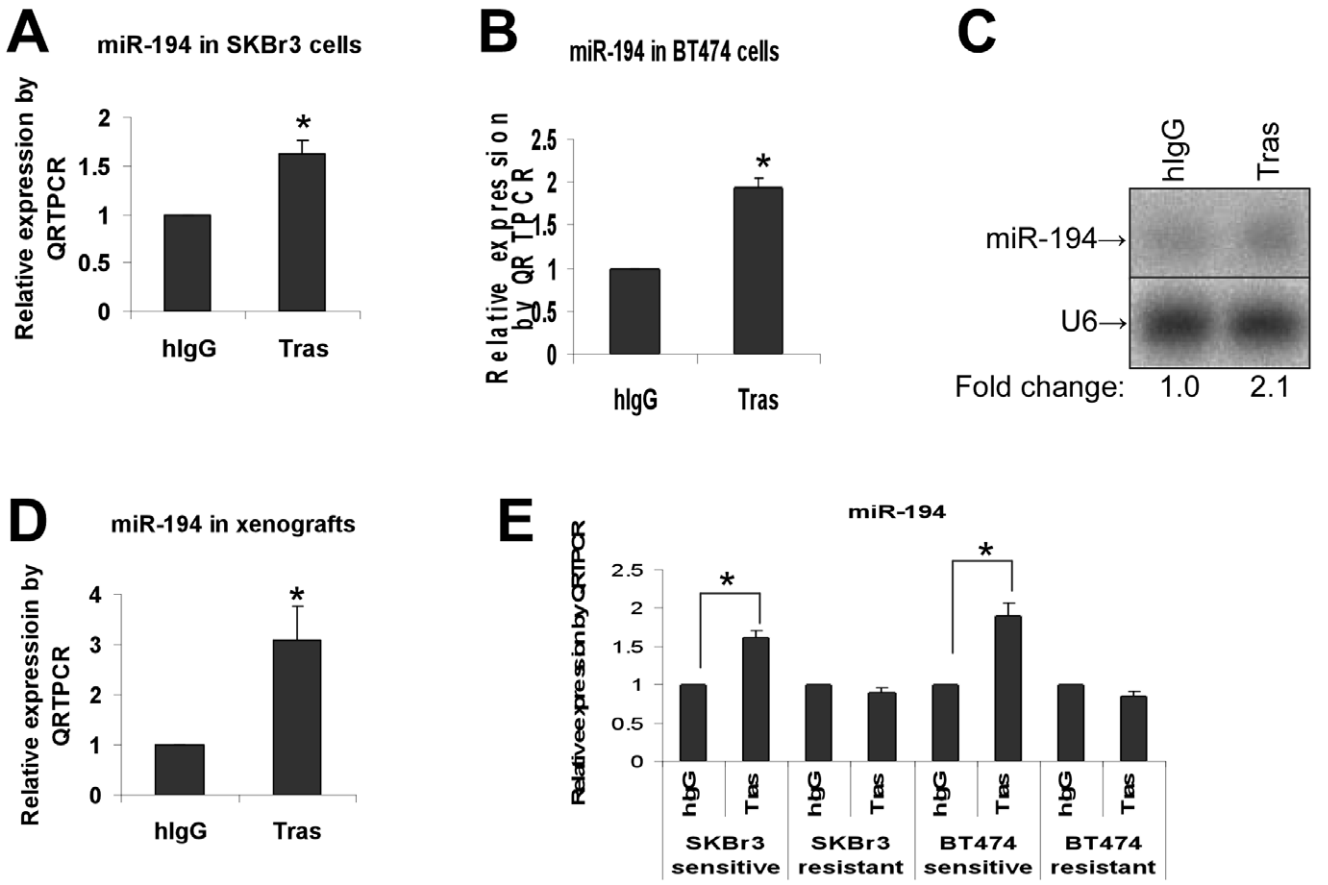

Figure 1. Trastuzumab upregulates miR-194 expression in vitro and in vivo. HER2-overexpressing breast cancer cell lines BT474 (**A**) and SKBr3 (**B**) were treated with trastuzumab (Tras) or control hIgG (10 μg/ml) for 48 hrs. Total RNA was prepared and analyzed by QRT-PCR to measure miR-194. * $p<0.05$ compared to hIgG control. (**C**) Northern blot analysis of miR-194 expression. BT474 cells were treated with Tras or control hIgG (10 μg/ml) for 48 hrs. Total RNA was prepared and analyzed by Northern blotting to detect miR-194. U6 non-coding small nuclear RNA (snRNA) served as a loading control. (**D**) QRT-PCR quantitation of miR-194 expression in vivo. BT474 xenografts in miR-194 levels were measured by QRT-PCR. * $p<0.05$ compared to hIgG control. (**E**) QRT-PCR quantitation of miR-194 expression in trastuzumab sensitive or resistant cell lines. Parental SKBr3 and BT474 (trastuzumab-sensitive), and their derived resistant cells were treated with Tras or control hIgG for 48 hrs. Total RNA was prepared and analyzed by QRT-PCR to detect miR-194. * $p<0.05$ compared to hIgG control.
doi:10.1371/journal.pone.0041170.g001

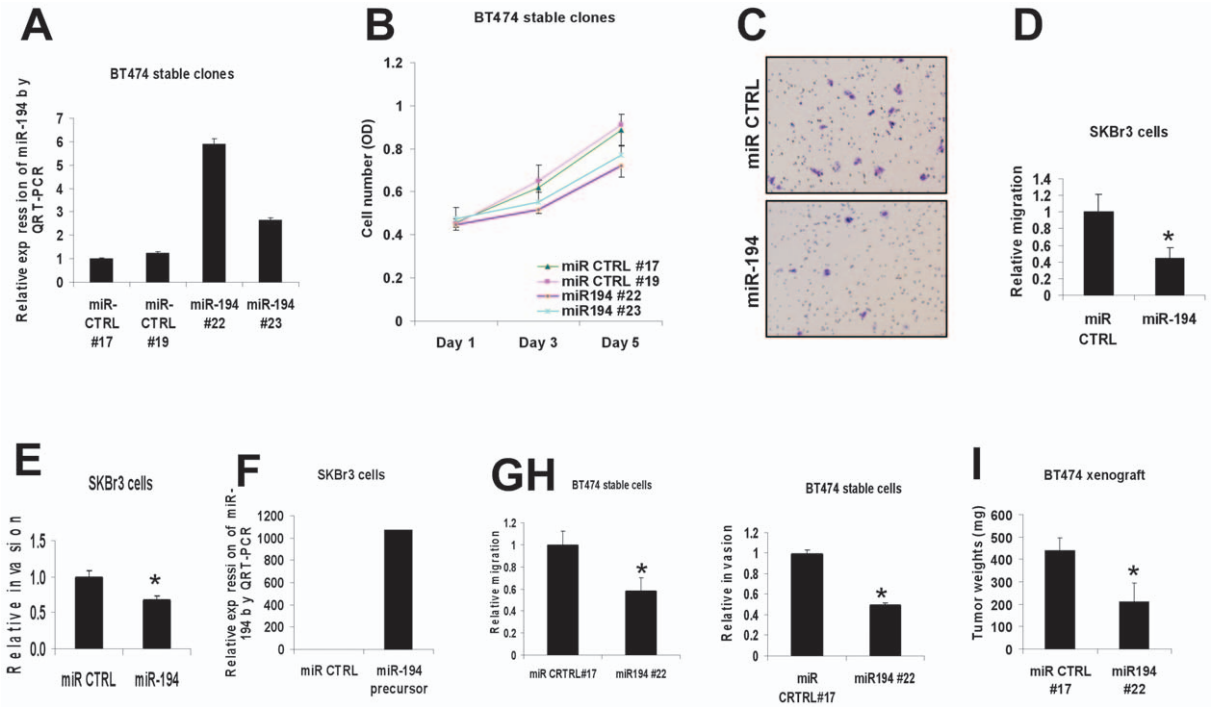

Figure 2. Increased miR-194 expression inhibits breast cancer cell migration and invasion. (**A**) miR-194 expression in the stable clones of BT474 cells. BT474 cells were stably transfected with an empty pEGFP-C1 vector or pEGFP-miR-194 vector under the selection of G418. Two control clones #17 and #19 that contain empty vector and two miR-194-expressing clones #22 and #23 were established and subjected for QRT-PCR analysis. Hsa-miR-194 was purchased from ABI (Assay ID 000493). (**B**) Cell viability assay of BT474 stable cells that express miR-194 or its control vector. BT474 cells were stably transfected with empty pEGFP-C1 vector or pEGFP-miR194 construct under the selection of G418. Two control clones #17 and #19 that contain empty vector and two miR-194-expressing clones #22 and #23 were chosen to measure viability of crystal violet-stained cells on day 1, day 3 and day 5. (**C**) Effect of miR-194 precursor on cell migration in SKBr3 cells. SKBr3 cells were transiently transfected with a miR-194 precursor or a control miRNA (miR CTRL) for 48 hrs and motility was measured overnight in a Transwell assay. (**D**) Quantitation of the SKBr3 cell migration as shown in (C). * $p<0.05$ compared to miR control. (**E**) Effect of miR-194 precursor on cell invasion in SKBr3 cells. SKBr3 cells were transiently transfected with a miR-194 precursor or a control miRNA (miR CTRL) for 48 hrs and invasion measured overnight. * $p<0.05$ compared to miR control. (**F**) miR-194 expression in transiently transfected SKBr3 cells. SKBr3 cells were transiently transfected with a miR-194 precursor or a control miRNA (miR CTRL) for 48 hrs. Total RNA was extracted and subjected to QRT-PCR analysis for miR-194 expression. Hsa-miR-194 was purchased from ABI (Assay ID 000493). (**G**) Assay of cell migration in BT474 stable cells that express miR-194 or a control vector. The control clone #17 and the miR-194-expressing clone #22 were chosen to study migration. * $p<0.05$ compared to #17 control. (**H**) Cell invasion assay in BT474 stable cells that express miR-194 or its control vector. The control clone #17 and the miR-194-expressing clone #22 were chosen to study invasion. * $p<0.05$ compared to #17 control. (**I**) BT474 xenograft tumor growth in vivo. BT474 xenografts in nude mice were established with the control clone #17 and the miR-194-expressing clone #22 as described in Methods. Tumors were collected and weighed after 4 weeks. * $p<0.05$ compared to #17 control.
doi:10.1371/journal.pone.0041170.g002

cytoskeletal proteins that concentrate in focal adhesions and link integrins to vinculin and actin [48,49]. To study the role of talin2 in cell migration, SKBr3 cells were transiently transfected with two siRNAs targeting talin2 or a negative control siRNA, and cell migration and invasion were assayed as described in Material and Methods. As shown in Figure 3D, knockdown of talin2 significantly inhibited migration of SKBr3 cells compared to the control siRNA. Talin2 silencing decreased cell invasion capacity as well (Fig. 3E). Two siRNAs used to downregulate talin2 was confirmed with Western blotting and shown in Figure 3F. Above data indicate that talin2 silencing decreases cell migration and invasion of SKBr3 cells.

Trastuzumab treatment inhibits cell migration and disrupts the normal vinculin staining pattern in breast cancer cells that overexpress HER2

While the effects of trastuzumab on cancer cell proliferation, angiogenesis, and apoptosis have been investigated in depth, the effect of trastuzumab on cell migration has received less attention. Both BT474 and SKBr3 cells respond to trastuzumab treatment, but are not actively motile. Long intervals are required to measure cell migration assay or to observe healing of scratches in cancer cell monolayers. Treatment of BT474 and SKBr3 with trastuzumab for 3 days significantly inhibited the ability of both cell lines to heal scratch assays (Fig. 4A). To study the effect of trastuzumab on cell migration over a shorter interval (16 h) and to minimize the effect of cell proliferation on scratch assays, we have stimulated SKBr3 cell migration with epidermal growth factor (EGF) and found that motility of trastuzumab-treated cells was significantly slower than that of hIgG-treated controls (Fig. 4B). Stimulation of SKBr3 cells with EGF for 16 hrs had no effect on miR-194 expression (data not shown). Similar results were observed in transwell cell migration assays (see below).

Treatment with trastuzumab disrupts cytosekeltal organization

SKBr3 cells were treated with trastuzumab and stained by immunofluorescence for expression of vinculin, a cytoskeletal protein associated closely with talin2 and exhibiting a similar subcellular distribution [49]. In the absence of a specific antibody for talin2 for immunofluorescence studies, we have used the

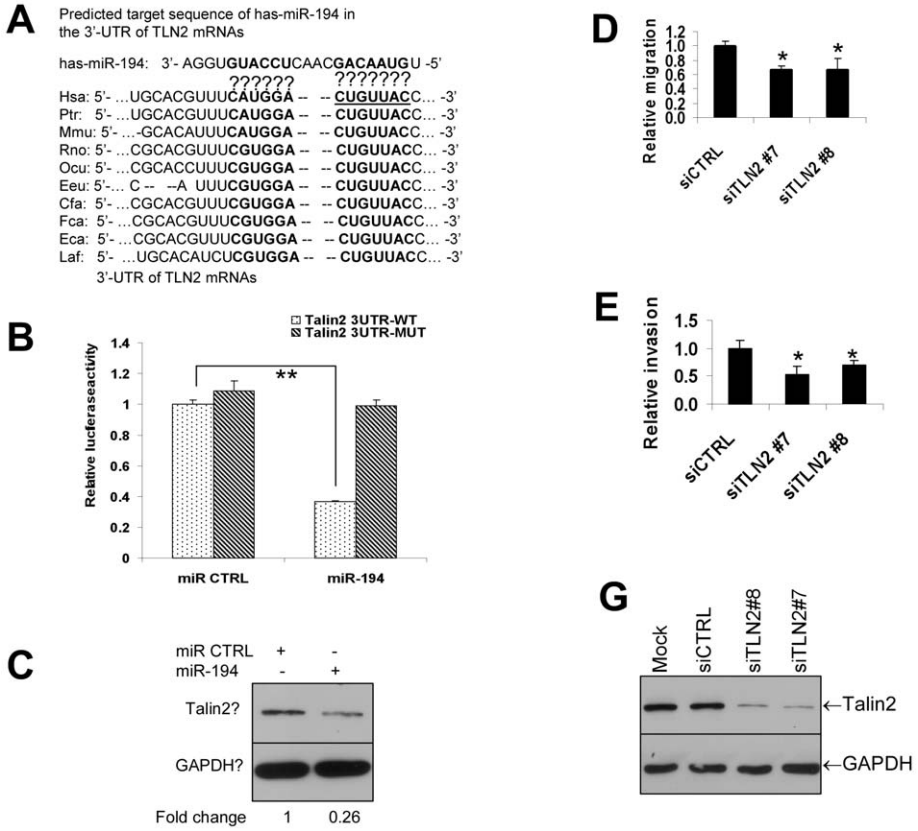

Figure 3. miR-194 targets the talin2 gene and downregulates talin2 protein levels. (**A**) Alignment of miR-194 with *talin2* (*TLN2*) 3′-UTRs. Complementary sequences of miR-194 and mammalian *talin2* 3′-UTRs are marked in Bold. The seed sequences of miR-194 are underlined. Has, human; Ptr, pan troglotydes; Mmu, mus musculus; Rno, rat; Ocu, rabbit; Eeu, hedgehog; Cfa, dog; Fca, cat; Eca, horse; Laf, elephant. The underlined seed nucleotides were deleted in the *talin2* 3′-UTR mutant reporter construct described in (B). (**B**) Effect of miR-194 expression on the luciferase activities of wild-type and mutated talin2 3′-UTR reporters. MDA-MB-361 cells were transiently transfected with a miR Control or miR-194 precursor for 36 hrs. Luciferase activity was determined using a dual luciferase assay. ** $p<0.01$. (**C**) Effect of miR-194 expression on talin2 protein levels in SKBr3 cells. SKBr3 cells were transiently transfected with a miR-194 precursor or a control miRNA (miR CTRL) for 48 hrs. Total protein was prepared and subjected to Western blotting. (**D**) Effect of talin2 downregulation on cell migration in SKBr3 cells. SKBr3 cells were transiently transfected with two siTalin2 (siTLN2 #7 and #8) or its control siRNA (siCTRL) for 48 hrs and then motility was measured overnight in a Transwell assay. * $p<0.05$ compared to siCTRL. (**E**) Effect of talin2 downregulation on cell invasion. SKBr3 cells were transiently transfected with two siTalin2 (siTLN2 #7 and #8) or its control siRNA (siCTRL) for 48 hrs and then invasion assay was performed. * $p<0.05$ compared to siCTRL. (**F**) Validation of talin2 siRNA efficacy. SKBr3 cells were transiently transfected with two siTalin2 (siTLN2 #7 and #8) or its control siRNA (siCTRL) or the transfection reagent only (mock) for 48 hrs and total protein was prepared. Western blotting was performed with a talin2 antibody.
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behavior of vinculin as a surrogate for talin2. As shown in Figure 4C, trastuzumab treatment for 16 hrs altered the staining pattern of vinculin. Instead of well ordered, discrete and focal staining of vinculin on the cell periphery, trastuzumab treatment produced disordered and clumpy staining both in the cytoplasm and at the cell's periphery. These observations suggest that trastuzumab can inhibit migration and disrupt the normal distribution of cytoskeletal proteins in breast cancer cells that overexpress HER2.

Trastuzumab treatment downregulates talin2 protein expression in vitro and in vivo in breast cancer cells that overexpress HER2

From the data presented above, trastuzumab treatment increases miR-194 (Tables 1 & 2 and Fig. 1) and miR-194 negatively regulates cell migration (Fig. 2) and talin2 expression (Fig. 3). To determine whether trastuzumab downregulates talin2 expression, BT474, SKBr3 cells and BT474 xenograft tumors were treated with trastuzumab or control hIgG and total protein was collected, Western blots prepared and stained with anti-talin2. As shown in Figure 4D, trastuzumab treatment decreased talin2 protein expression in a dose-dependent manner in BT474 cells. Quantitation of talin2 protein levels after trastuzumab treatment in three different experiments confirmed the dose-dependent effect (Fig. 4E). Trastuzumab treatment also decreased talin2 protein in SKBr3 cells (Fig. 4F). A similar downregulation of talin2 was observed in BT474 xenografts growing in nude mice. Trastuzumab inhibited talin2 protein expression (the lower band) in two different tumors (Fig. 4G). Trastuzumab treatment also produced a decrease in total Rb protein in BT474 xenografts, consistent with activity of the antibody in vivo [15].

Knockdown of miR-194 promotes cancer cell migration and reverses trastuzumab inhibition of cell migration

To determine whether a decrease in miR-194 would increase motility, SKBr3 cells were transiently transfected with a specific miR-194 inhibitor or antagomir. As shown in Figure 5A, transfection of SKBr3 cells with a miR-194 antagomir increased

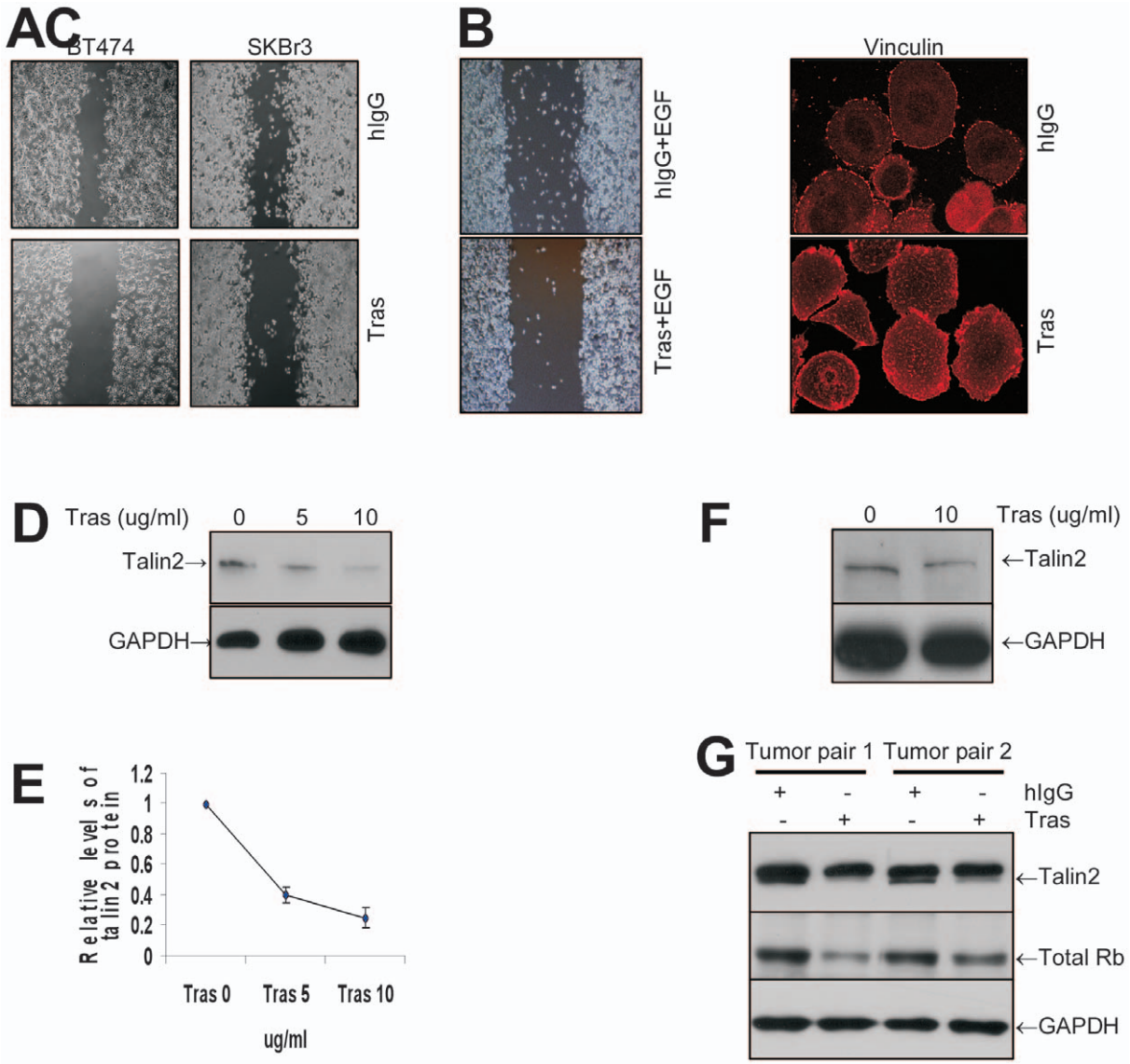

Figure 4. Trastuzumab treatment downregulates talin2 protein expression and inhibits breast cancer cell migration. (**A**) Effect of trastuzumab (Tras) on cell migration in a scratch assay. BT474 and SKBr3 cells were seeded in 6-well plates. After cells had grown to confluence, a scratch was made in the monolayer. Cells were treated with trastuzumab (Tras) or control hIgG (10 μg/ml) for 72 hrs. Images were recorded at 72 hrs at 40× enlargement. (**B**) Effect of trastuzumab on cell migration over a shorter interval (16 h) in a scratch assay. SKBr3 cells were seeded in 6-well culture plates and cultured overnight to achieve a cell density of full confluence. A scratch was made in the monolayer. Cells were then treated with trastuzumab (Tras) or control hIgG (10 μg/ml) plus epidermal growth factor (EGF, 20 ng/ml, sigma) for 16 hrs. Images were recorded at the end of 16 hrs of EGF stimulation at 40× enlargement. (**C**) Effect of trastuzumab (Tras) on cytoskeletal vinculin distribution after IF staining. SKBr3 cells were treated with trastuzumab (Tras) or control hIgG (10 μg/ml) for 48 hrs, and then subjected to IF staining as described in Methods. (**D**) Effect of trastuzumab on talin2 protein in BT474 cells. BT474 cells were treated in vitro with different concentrations of trastuzumab for 48 hrs. Total protein was prepared and subjected to Western blotting. (**E**) Quantitation of talin2 expression. The talin2 bands on immunoblots from three different experiments including the one shown in (**D**) were digitized, normalized to the levels of GAPDH, and expressed as mean levels (error bars correspond stand deviation). The talin2 expression at trastuzumab 0 concentration was set at 1. (**F**) Effect of trastuzumab on talin2 protein in SKBr3 cells. SKBr3 cells were treated with trastuzumab (Tras) or control hIgG (10 μg/ml) for 48 hrs. Total protein was prepared and subjected to Western blotting. (**G**) Effect of trastuzumab on talin2 protein in BT474 xenografts. BT474 xenografts in nude mice were treated with trastuzumab (Tras) or hIgG 1 mg/kg intraperitoneally twice a week and for 3 weeks. Total cell lysates were prepared and subjected to Western blotting.
doi:10.1371/journal.pone.0041170.g004

cancer cell migration by more than 55%. The ability of the miR-194 antagomir to silence miR-194 was confirmed by QRT-PCR as shown in Figure 5B. Similar results were observed in BT474 cells, where inhibition of miR-194 by a miR-194 antagomir stimulated BT474 cell migration (Fig. 5C). Consistent with the results of *in vitro* scratch assay, trastuzumab treatment decreased the BT474 cell migration (Fig. 5C). However, trastuzumab treatment was no longer able to inhibit cell migration in the presence of miR-194 antagomir or inhibitor (Fig. 5C), indicating miR-194 induction was required for trastuzumab to slow cell migration in breast cancer cells.

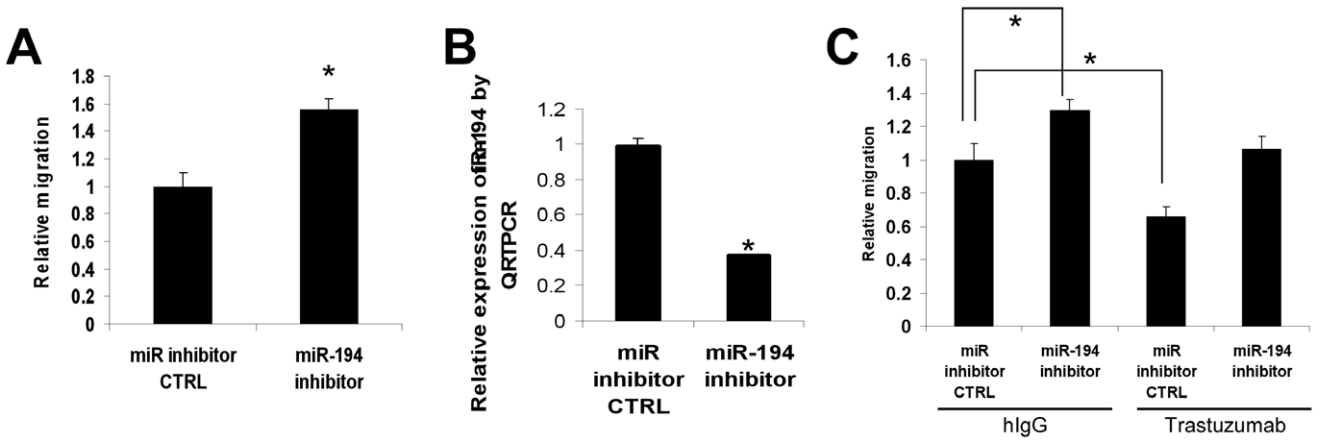

Figure 5. miR-194 inhibitor stimulates cell migration and blocks trastuzumab-inhibited cell migration. (**A**) Effect of miR-194 inhibitor on cell migration in SKBr3 cells. SKBr3 cells were transiently transfected with a miR-194 inhibitor or its negative control (miR inhibitor CTRL) for 48 hrs before measurement of migration overnight. * $p<0.05$ compared to the negative control. (**B**) Validation of efficacy of miR-194 inhibition. SKBr3 cells were transiently transfected with a miR-194 inhibitor or its negative control (miR inhibitor CTRL) for 48 hrs. Total RNA was prepared and miR-194 measured by QRT-PCR. * $p<0.05$ compared to miR inhibitor control. (**C**) Effect of miR-194 inhibitor on trastuzumab-inhibited cell migration. SKBr3 cells were transiently transfected with a miR-194 inhibitor or its negative control (miR inhibitor CTRL) for 16 hrs, treated with trastuzumab (Tras) or control hIgG (10 μg/ml) for 36 hrs, and motility was then measured for overnight in Transwell assays. * $p<0.05$.
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Discussion

In this study, we have found for the first time that miR-194 is induced in HER2-overexpressing breast cancer cells by trastuzumab treatment. Forced expression of miR-194 inhibits cell migration and decreases levels of talin2, a cytoskeletal protein. Treatment with trastuzumab also inhibits breast cancer cell migration and decreases talin2 expression. Depletion of miR-194 stimulates breast cancer cell migration and abolishes trastuzumab-inhibited cell migration.

The regulatory role of miR-194 was first studied in normal and malignant cells of the gastrointestinal tract. High levels of miR-194 are expressed in the intestines and liver [50,51]. Hepatocyte nuclear factor (HNF) can induce miR-194 expression during intestinal epithelial cell differentiation [52,53]. miR-194 suppresses invasion and migration of liver mesenchymal-like cancer cells [54]. miR-194 expression is elevated in normal colon tissues and low in colon cancers [55]. Low miR-194 expression has been associated with large tumor size and advanced stage in gastric cancer [56]. Activation of tumor suppressor p53 can induce miR-194 and its clustered miR-192 and miR-215 expression [55,57]. miR-192 and miR-215 can induce p21Cip1 and cell cycle arrest in colon cancer cells [55]. In endometrial cancer cells, miR-194 has been reported to inhibit self-renewal factor BMI-1, reduce cell invasion and inhibit epithelial-mesenchymal transition (EMT) [58]. While inhibition of cell migration by miR-194 has been demonstrated in other systems, this is the first report to show that miR-194 can inhibit breast cancer cell migration (Fig. 2) and that miR-194 and migration can be regulated by trastuzumab.

Our data suggest that miR-194-induced inhibition of motility is mediated by downregulation of cytoskeletal proteins. We have demonstrated for the first time that miR-194 inhibits talin2 protein expression and binds to the talin2 3′-UTR (Fig. 3). In addition to regulating talin2, we have also observed that miR-194 expression reduces profilin2, another cytoskeletal protein (data not shown). However, miR-194 did not bind directly to the profilin2 3′-UTR in a luciferase reporter assay. miR-194 has been shown by others to target migration-related proteins including N-cadherin, Rac1, heparin-binding epidermal growth factor–like growth factor, type 1 insulin-like growth factor receptor, protein tyrosine phosphatase-non receptor type 12, integrin-alpha 9, suppressor of cytokine signaling 2, and BMI1 polycomb ring finger oncogene [54,56,58]. Inhibition of these other cytoskeletal and migration-related proteins may contribute to miR-194-induced inhibition of cell migration/invasion as well.

Talin1 and talin2 are actin-binding cytoskeletal proteins that play key roles in cell signaling, adhesion, and migration [48,49,59,60]. Both talin1proteins are closely coupled to vinculin, another cytoskeletal protein found in focal adhesion complexes [49]. Both talin1 and talin2 (74% identity) can bind to integrins and to F actin via FERM (four-point-one, ezrin, radixin, moesin) domains and are important regulators of integrin activation [59,60,61,62]. Genetic knockout of talin1 alone does not affect fibroblast spreading due to the compensatory activity of talin2, but does impair cytoskeletal organization [63]. Double deletion of talin1 and talin2 abolishes extracellular matrix–cytoskeletal linkage through integrins and blocks assembly of focal adhesions [64]. Talin1 knockdown in the absence of talin2 compensation has been shown to prevent spreading of endothelial cells and block angiogenesis leading to embryonic death [65]. Forced expression of talin1 enhances migration and invasion of prostate cancer cells [66]. Talin1 overexpression is a poor prognostic factor in prostate [66], liver [67] and oral [68] cancers. The role of talin2 in cancer cells including in breast cancer has not yet been reported. This study indicates that suppression of talin2 can inhibit breast cancer cell migration.

Trastuzumab treatment seems to exert its anti-tumor effect through multiple mechanisms including inducing miRNAs. After completing this study, one report has been just published and shown that trastuzumab treatment can induce miR-26a and miR-30b, leading to inhibition of cylcin E2 expression and G1 arrest of the cell cycle [69]. Our study has identified another miRNA (miR-194) and new target (talin 2, motility) in HER2-overexpressing breast cancer cells.

In conclusion, our study describes a novel mechanism of trastuzumab action in breast cancer cells. Treatment with trastuzumab can activate miR-194 expression, downregulate

cytoskeletal protein talin2 expression and inhibit cell migration/invasion in HER2-overexpressing breast cancer cells.

Supporting Information

Figure S1 Heatmap of the differentially expressed miRNAs in response to trastuzumab treatment in HER2-overexpressing SKBr3 cells (A) and BT474 cells (B). To visualize the clusters of expressed miRNAs after trastuzumab treatment, hierarchical clustering was performed based on the differentially expressed miRNAs. Names of samples (top) and miRNAs (side) is shown by using One Minus Correlation as a distance measure after genes centering and scaling, and average linkage method for defining the distance between the clusters. Sample BRC20, SKBr3 cells treated control hIgG for 20 hrs; BRHCT20, SKBr3 cells treated trastuzumab for 20 hrs; BTC48, BT474 cells treated with control hIgG for 48 hrs; BTHCT48, BT474 cells treated with trastuzumab for 48 hrs. A color scale of expression is shown between panel A and B.
(TIF)

Figure S2 Effects of trastuzumab on miR-194 expression in the MDA-MB-231 cells with low-HER2 level and the KPL4 cells with high-HER2 level. Two trastuzumab-insensitive breast cancer cell lines MDA-MB-231 (**A**) and KPL4 (**B**) were treated with trastuzumab (Tras) at different concentrations (0, 5, 10 μg/ml) for 48 hrs. Total RNA was prepared and analyzed by QRT-PCR in triplicate for miR-194 levels.
(TIF)

Author Contributions

Conceived and designed the experiments: XFL GAC RCB. Performed the experiments: XFL MIA WM RS MSN SZ. Analyzed the data: XFL SR. Contributed reagents/materials/analysis tools: YW GAC. Wrote the paper: XFL RCB.

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